Different clinical outcomes of tuberculosis can be related to the balance between cell-mediated and humoral immunity. In this prospective study we examined the humoral immune responses to recombinant and native mycobacterial antigens in relation to clinical presentations of pulmonary TB. Two hundred and fifteen serum samples were examined including: non-cavitary (n = 120), cavitary (n= 65), caseous pneumonia (n=12), and disseminated TB (n=18). ELISA tests detecting IgG, IgA, and IgM against antigens: 38kDa and 16kDa, 38kDa and lipoarabinomannan (LAM) were used. Univariate and multivariate logistic regression analyses were carried out to find the association between the antibody level and demographic or clinical characteristics. The relationships among specific antibody profiles and the phase of the disease in relation to demographic (age and sex) and clinico-radiological factors were investigated by measuring serum antibody levels (IgG, IgA, and IgM) to 38kDa and 16kDa recombinant M. tuberculosis antigens and to LAM – native mycobacterial antigen. The results show that the radiological extent of the disease is the strongest factor associated with IgG antibody production. Patients with more extensive pulmonary TB showed higher titers of IgG antibody to M. tuberculosis antigens (P<0.0001). The highest IgG and IgA level were observed in fibro-cavernous TB. The presence of cavity was associated only with IgG anti 38+16kDa (P<0.001). IgA level was the highest in caseous pneumonia. IgM antibody production was not associated with any clinical and radiological factor, but only with the male gender. Age was independently and inversely associated with IgG anti 38kDa+LAM level and IgM anti 38kDa+LAM. We conclude that the humoral immune response to mycobacterial antigens is highly heterogeneous and varies with the stage of TB. IgG antibody level is higher in most advanced and extensive forms of the disease.

**Key words:** humoral immune response, mycobacterial antigens, tuberculosis
INTRODUCTION

*M. tuberculosis* infection presents in various clinical forms, ranging from asymptomatic lifelong infection to multibacillary disseminated disease (1, 2). Within the framework of contemporary immunology, different patterns of the host response suggest the existence of a broad spectrum of immune reactions to *M. tuberculosis*. The spectrum of TB is compared to leprosy, with an inverse relationship between the antibody and T-cell immunity (2-5). At one pole of the spectrum, in self-limiting disease, patients mount a strong Th1 type immune response and localize the infection (3-5). At the opposite pole, in disseminated form, there is diminished cell-mediated immunity and augmented antibody response, which results in failure to restrict the growth of the pathogen (4-6). However, in human TB the striking correlation between the ability to generate the cell-mediated immune response and to restrict the growth of *M. tuberculosis* has not been established.

Although the immunity to TB is cell-mediated, humoral responses are common (4-7). The dogma has been that the antibody response is unimportant for TB immunity and pathogenesis (7). The inability of various investigators to generate effective sera in prevention and treatment of TB infection was an important contributor to today’s belief that the antibody-mediated immunity plays a minor or negative role in the outcome of TB infection (7). However, majority of published experiments with the use of serum therapy for TB did produce some evidence of the efficacy of the serum against *M. tuberculosis* (7). Unfortunately, lack of appropriate controls, minimal descriptions in many studies, and unknown antibody levels make a large part of the experience with serum therapy inconclusive in regard to the role of antibody in protection against TB (7).

The knowledge of the humoral immune responses at various stages of TB infection and disease could help elucidate the complex interaction between the host and the pathogen (4-7). A comprehensive immune profiling of antigen-specific responses in relation to clinical phase of the disease is critical not only to the understanding of disease pathogenesis, but this knowledge can be used for diagnostic purpose. Therefore, in the present study we attempted to correlate the type and intensity of the humoral immune response to mycobacterial antigens with different clinical manifestations of pulmonary TB.

MATERIAL AND METHODS

Patients and tuberculosis status

Serum samples from 215 subjects with pulmonary TB (78 females and 137 males) were examined. The mean age of the examined group was 44.7 years (range 19-90 years). TB patients were referred to the Institute of Tuberculosis and Lung Diseases in Warsaw, Poland and Tuberculosis and Lung Diseases Center in Otwock, Poland. All subjects gave informed consent for participation in the study and the study protocol was approved by a local Ethics Committee.
patients were Caucasian, HIV negative, and previously BCG vaccinated. Patients were classified into four groups depending on the results of clinical and radiological findings: (1) 120 cases of non-cavitary infiltrative TB, (2) 65 cases of cavitary disease, (3) 12 cases of caseous pneumonia and (4) 18 cases of disseminated TB (miliary TB). According to the radiological extend of the disease, all patients were also categorized into 3 groups: minimal changes (disease limited to 1 lobe) (n=78), moderate changes (bilobular changes) (n=68), and advanced disease (changes in 3 or more lobes) (n=69). All patients with advanced disease had bilateral disease or miliary TB. All cases represented postprimary form of tuberculosis. The group examined included 150 new cases (duration of symptoms shorter than 6 months), 51 chronic cases with multidrug resistant TB, and 68 cases of recurrent TB. Recurrent TB was defined as the confirmed TB episode 6 months or more after the completion of previous antituberculous therapy that was considered successful. The diagnosis was confirmed by sputum culture in 133 from new and recurrent cases and in all (n=51) chronic cases. The examination of acid-fast bacilli was made using Ziehl-Neelsen stain, culture on Löwenstein-Jensen solid egg-based medium or by Bactec system. In 12 patients, the diagnosis was confirmed by histological testing (evidence of caseating granulomas in pleural biopsy specimen). In 19 patients, TB was diagnosed by panel decision on the base of suggestive clinico-radiological signs (in the absence of evidence for alternative infective or non-infective process), tuberculin skin test, and reevaluation after specific antituberculous treatment. The evidence of pleural tuberculosis was present in 14 patients. A hundred and eighty three patients did not receive antituberculous treatment at the time of blood collection, and in the 32 remaining cases the duration of therapy was shorter than 1 month. Six patients received oral corticosteroids (pleural involvement, disseminated TB).

**Methodology**

Blood samples were collected, centrifuged, and the serum was stored at -40°C until use. An array of commercial immunoenzymatic kits to detect IgG antibodies against 38kDa plus 16kDa (Pathozyme tb complex plus, Omega Diagnostics, Scotland) and IgG, IgA, and IgM antibodies to 38kDa plus lipoarabinomannan (LAM) (MycoG, MycoA, and MycoM, Omega Diagnostics, Scotland) was applied. 38kDa and 16kDa are recombinant mycobacterial antigens expressed in and purified from *E. coli*. LAM is a native mycobacterial antigen. All tests are based on a solid double antibody sandwich ELISA. Sera diluted 1:50 or 1:100 (according to the manufacturer’s instruction) were added to microwells precoated with antigens. All samples were assayed in duplicates. In the positive cases, antigen-antibody complex reacted with peroxidase-labeled antihuman IgG (IgA or IgM) conjugate. Using H$_2$O$_2$/TMB as substrate, the enzymatic activity of peroxidase was measured at 450 nm with the use of automated reading system ELX 800 (Biotec). All the results except for IgM tests were referred to the standard curve. The standards were provided for the generation of a semi-logarithmic reference curve. As the sera were diluted 1:50 or 1:100, the units extrapolated from the standard curve were multiplied by 50 (100) to obtain sero-units for the result interpretation. Results of IgM tests were expressed as the ratio of optical density (OD) of the examined sample to the OD of a cut-off sample.

**Statistical analysis**

Results of antibody titers were compared by analysis of variances (ANOVA). Univariate analysis and multivariate regression logistic analysis were performed to analyze variables that might have influenced the humoral immune response to the examined antigens. All variables of the same category that were significant in the univariable analysis were compared with the use of the Tukey multiple comparison test for pairwise comparisons. Statistical significance was accepted at a level of P<0.05. All variables whose changes appeared significant by univariate analysis were subjected to
the multivariable analysis. Results of multivariate analysis (all categories together) are expressed as adjusted regression coefficients (CR) with their associated 95% confidence intervals (CI). Non-significant variables were eliminated. All analyses were performed with S-PLUS 2000 (8).

RESULTS

In univariate analysis, the IgG response to 38+16kDa was associated with: culture positive TB, chronic TB, fibro-cavernous or disseminated disease, and the presence of the cavity and extensive radiological changes. In the culture positive cases, the mean antibody level was 437.15 U/ml and in the culture negative cases - 163.21 U/ml (P<0.001). The mean antibody level in new cases was 313.97 U/ml, in chronic cases – 775.19 U/ml, and in recurrent disease – 334.47 U/ml. The difference between new and chronic cases was statistically significant (P<0.001). The mean antibody levels in different clinical presentation of the disease were the following: infiltrative TB – 312.64 U/ml, disseminated TB – 640.15 U/ml, caseous pneumonia – 218.68 U/ml, fibro-cavernous disease – 584.47 U/ml. Statistical differences between infiltrative and disseminated or fibro-cavernous form were significant (P<0.001). In the patients with radiological changes limited to 1 lobe, the mean antibody level was 91.71 U/ml, with bilobular changes – 424.82 U/ml, and with extensive changes – 674.79 U/ml. All groups

![Fig. 1. Mean IgG anti-38+16kDa level (±SD) in relation to examined factors. Fib-cav – fibrocavernous TB, Pneu cas – pneumonia caseosa, Dissem – disseminated TB, Inflitr – infiltrative TB; Hist – diagnosis confirmed by histological testing, Clin/rtg – diagnosis on the base of clinico-radiological criteria, BA – culture positive TB; Recurr – recurrent TB, Chronic – chronic cases, New – new cases; 1 areas – 1 lobe involvement, 2 areas – 2 lobes involvement, 3 areas – 3 and more lobes involved; Caverna Y – cavitary TB, Caverna N – non-cavitary TB; M - men, W - women.](image)
differed significantly (P<0.0001). In the cavitary form, the antibody level was 543.46 U/ml and in the non-cavitary form - 192.96 U/ml (P<0.001). These results are presented in Fig. 1.

The IgG response to anti-38kDa+LAM was associated with the phase of the disease and its radiological extent. The mean antibody level in infiltrative TB was 331.77 U/ml, in disseminated form – 140.60 U/ml, in caseous pneumonia – 885.14 U/ml, and in fibro-cavernous TB – 620.27 U/ml. The difference between infiltrative and fibro-cavernous disease was statistically significant (P=0.003). The mean antibody level in the group with minimal radiological changes was 247.27 U/ml, with moderate changes - 444.35 U/ml, and with extensive changes – 612.29 U/ml. The difference between the groups with minimal and extensive changes was significant (P<0.01) (Fig. 2). IgG anti-38kDa+LAM level was inversely correlated with age (P<0.001) (data not shown).

The IgA anti-38kDa+LAM response was associated only with the radiological extent of TB: 1 lobe changes – 340.19 U/ml, bilobular – 864.21 U/ml, and extensive changes – 798.12 U/ml. The difference between the groups with minimal and extensive changes was significant (P=0.02) (Fig. 3).

The production of IgM anti-38kDa+LAM was associated only with the gender. The mean OD index in the group of men was 0.72 and in group of women was 1.09 (P=0.02) (Fig. 4). IgM anti-38kDa+LAM was also negatively correlated with age (P<0.01) (data not shown).

On the basis of multivariate logistic regression, independent factors associated with the humoral response to specific antigens were identified. The results are presented as adjusted regression coefficients (CR) with their associated 95%
confidence intervals (CI). Results higher than 1 indicate that the category selected presents with a higher mean antibody level than the reference category. Results lower than 1 indicate an inverse relation between the categories compared.

The radiological extent of changes was associated with the IgG level anti-38+16kDa (bilobular changes - CR=2.15, P<0.01, 3 or more lobes involved - CR=4.66, P<0.001) and with the IgG anti 38kDa+LAM (bilobular changes - CR=1.6, P=0.04, 3 or more lobes involved - CR=4.66, P<0.01). The reference category was taken as the disease limited to 1 lung lobe. Clinical course of the disease was independently associated with IgG anti-38+16kDa (category chronic cases - CR=2.36, P<0.05); the reference category being new cases. Radiological presentation of the disease was associated with IgG anti 38kDa+LAM antibody level (fibro-cavernous - CR=1.98, P<0.02) and IgA anti-38kDa+LAM (caseous pneumonia - CR=4.13, P<0.01); the reference category being infiltrative TB. The male gender was independently associated with lower IgM level (CR=0.71, P<0.01). Age was independently associated with IgG anti-38kDa+LAM level (CR=0.98, P<0.001) and IgM anti-38kDa+LAM (CR=0.99, P=0.04). The correlation was negative in both situations. Fig. 5 presents the results of multivariate analysis. These results are expressed as a relative risk. Lower and higher limits of each variable determine the confidence interval.

Fig. 3. Mean IgA anti-38kDa+LAM level (±SD) in relation to the examined factors. Abbreviations as in Fig. 1.

Fig. 4. Mean optical density index (±SD) of IgM anti-38kDa+LAM measurement in relation to the examined factors. Abbreviations as in Fig. 1.
CR=1.6, P=0.04, 3 or more lobes involved - CR=4.66, P<0.01). The reference category was taken as the disease limited to 1 lung lobe. Clinical course of the disease was independently associated with IgG anti-38+16kDa (category chronic cases - CR= 2.36, P<0.05); the reference category being new cases. Radiological presentation of the disease was associated with IgG anti 38kDa+LAM antibody level (fibro-cavernous - CR= 1.98, P<0.02) and IgA anti-38kDa+LAM (caseous pneumonia - CR= 4.13, P<0.01); the reference category being infiltrative TB. The male gender was independently associated with lower IgM level (CR=0.71, P<0.01). Age was independently associated with IgG anti-38kDa+LAM level (CR=0.98, P<0.001) and IgM anti-38kDa+LAM (CR=0.99, P=0.04). The correlation was negative in both situations. Fig. 5 presents the results of multivariate analysis. These results are expressed as a relative risk. Lower and higher limits of each variable determine the confidence interval.

DISCUSSION

It is generally accepted that the immune response to M. tuberculosis is biphasic, with the IFN-γ cellular response detected early and IL-4 driven antibody response detected late in the course of infection (9-11). In the present study, the humoral immune response to the M. tuberculosis antigens at different stages of disease was evaluated. The relationships between the specific antibody profiles and the phase of the disease and in relation to demographic (age and sex) and clinico-radiological factors (culture status, radiological extent, clinical form of the disease, etc) was investigated by measuring the serum antibody levels (IgG,
IgA, and IgM) to 38kDa and 16kDa recombinant *M. tuberculosis* antigens and to LAM-native mycobacterial antigen.

Age was independently and inversely associated with the IgG anti-38kDa+LAM and IgM anti-38kDa+LAM levels. IgM antibody is the first to appear in response to any antigen and therefore could be expected to be high in recent TB and to decline in most advanced phases as a result of longer duration of infection in older age (12). Epidemiological data from Poland confirm that the incidence of TB is higher in older age groups. A decline of IgG antibodies with age is probably related to aging of the immune system (12). It is probably dependent on T-helper function impairment in older age, resulting from thymic involution (12). A deficit of T helper cells leads to impairment of B cell function and lower production of specific antibodies (12). The IgM level was 1.4 time higher in women than in men. That may be related to different clinical presentations of the disease in either gender, but this kind of analysis was not performed in the present study. On the other hand, IgM antibody production was not associated with any clinical and radiological factor, as opposed to IgG and IgA production. Several authors suggested that IgM are produced mainly in the early phase of primary TB infection (13, 14). We were not able to confirm this observation as most of our patients presented with post-primary form of the disease. In our study, the early and late phases of post-primary TB did not differ with respect to the IgM production. It is known that the IgM synthesis is regulated by different mechanisms than those responsible for synthesis of other classes of immunoglobulin, which are generally T-cell independent. IgM antimycobacterial antibodies may be influenced by environmental factors, such as BCG vaccination, contact with environmental mycobacteria, or even the presence of latent TB infection (14).

In the present study, several associations between the type and intensity of the humoral immune response to mycobacterial antigens and different manifestations of pulmonary TB were found. Chronic tuberculosis was positively associated with IgG anti 38+16kDa compared with new TB cases. This observation can be explained by a long-term stimulatory effect and intensity of stimulation in chronic cases. Chronic TB patients are usually non-compliant, with multidrug resistant disease, irregularly treated. Several patients from the examined group died in the course of drug resistant TB. It is possible that an increased antigenic load in this group of patients caused persistent and intensive stimulation of antibody production along with suppression of the cellular arm of the immune response. We found no data in the literature concerning the humoral immune response in chronic TB patients. In the present study, IgG antibody titer was higher in culture positive groups. This observation was confirmed by others (14, 15). Although the IgG level was higher in culture positive groups, compared with culture negative ones, the culture status surprisingly had no independent impact on the serologic response. The strongest factor independently associated with the intensity of antibody production was the radiological extent of TB changes. This concerned
both IgG and IgA-based tests. The humoral IgG response was highest in most extensive radiological cases, but the difference between the group with minimal changes (1 lobe involvement) and the group with moderate changes (2 lobes) was also highly significant. The humoral immune response based on IgG anti-38+16kDa and IgG anti-38kDa+LAM was more than 4 times stronger in advanced compared with the minimal TB changes group. Similar observations have been noted by other authors (14, 16-18). Radiological extensiveness of the disease is the strongest factor associated with antibody production. Progress of the disease in most advanced forms may result from diminished local cellular response. An impairment of T-cell function leading to dissemination of the disease is also observed in immunosuppressed patients. However, in such cases a weak T-cell response is also connected with deficient antibody production. An augmented humoral response may accompany slow, long-term progression of the disease in untreated immunocompetent patients. In such situations, IgG and IgA-based immune response is pronounced in parallel with persistent stimulation due to increasing antigenic load. Progression of the disease may also be explained by the hypothesis of polarization of immune response, causing a shift toward the humoral arm that is not able to combat the disease (19). This is an open question if such imbalance is a primary phenomenon followed by the progression of the disease, or the other way around. A correlation between antibody production and infection stage together with genetic background was found in animals infected with *M. tuberculosis* (15-17, 19). In the present study, selected radiological presentations of the disease were associated with IgG and IgA, but not with IgM, production. The IgG and IgA levels were lowest in infiltrative TB compared with all other examined presentations. The highest IgG and IgA levels were observed in fibro-cavernous TB, which is an advanced, post-primary form of the disease. Similar findings have been reported by other authors (14, 15, 18). Increased production of IL-4 in TB patients has been observed in individuals with advanced involvement of lung parenchyma, with high bacterial loads in sputum (20). In our study, the presence of cavity was associated only with IgG anti-38+16kDa. This may be due to the fact that in this group there were patients with advanced (fibro-cavernous TB) and also those with an early form of TB (infiltrative TB with necrosis). These results suggest that an alteration in type 1 and type 2 cytokine balance can occur in TB patients being in advanced clinical stage.

The clinical spectrum of TB is related directly to the strength of activation of various arms of the immune system. Patients with self-limiting TB show strong T cell reactivity, while patients with the disseminated form of the disease present an augmented antibody response. IgG antibodies (interleukin 4-dependent) correlate best with clinical and radiological stage of the disease. Patients with more extensive pulmonary tuberculosis showed higher titers of IgG antibody to *M. tuberculosis* antigens. IgG antibodies to 38 kDa antigen are elicited during the advanced stage of the disease (21, 23). The Th1-type immune response is believed to be necessary for protection against mycobacterial pathogens, such as
Mycobacterium tuberculosis (5, 6, 21). These facultative intracellular pathogens reside in mononuclear cells, which allow them to escape the immune response of the host. Therefore, there is a crucial requirement for a coordinated cellular immune response to control the infection. Humoral immunity is strong only in the presence of large numbers of microorganisms and vary depending on the specificity of the antibodies. Early tuberculosis and advanced tuberculosis are characterized by different antibody profiles. It indicates that the targets of the immune response are related to the tuberculosis state and the intensity and duration of infection (22-24). Further insights into the origin of the unique immune spectrum in TB require a detailed analysis of the cellular and humoral immune mechanisms.

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