

Antibody repertoire against the A60 antigen complex during the course of pulmonary tuberculosis

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Antibody repertoire against the A60 antigen complex during the course of pulmonary tuberculosis. M. Amicosante, G. Paone, F. Ameglio, E.L. Bianchi, E. Piccolella, L. Richeldi, A. Bisetti, M. Luisetti, C. Saltini. ©ERS Journals Ltd., 1993.

ABSTRACT: The A60 antigen complex is a *Mycobacterium bovis* (BCG) highly immunodominant antigen containing both B and T-cell epitopes. Clinical-serological studies show that elevated anti-A60 titres are present during tuberculosis. We wished to analyze in detail antibody responses against A60 components during the course of tuberculosis.

A mixed longitudinal study was designed including individuals at the onset of tuberculosis, during treatment and after resolution of the disease. The anti-A60 repertoire was analyzed using a western blot assay with A60 as the antigen.

While PPD⁻ normals recognized only the 65 kDa heat shock protein (HSP), PPD⁺ normal individuals displayed low levels of anti-A60 antibodies against dominant antigens. These were immunoglobulin M (IgM) and immunoglobulin G (IgG) consistent with response to a latent infection. Onset tuberculosis was characterized by IgM and IgG antibodies against 52 to 28 kDa antigens; IgM response being limited to earlier phases of the disease. In contrast, IgM antibodies against 25 to 14 kDa antigens appeared only 2-6 months after disease onset. The antibody repertoire of chemotherapy-treated, resolved tuberculosis was exclusively IgG in isotype, as for a memory-type response.

Thus, western blot analysis with A60 identifies typical antibody patterns associated with different clinical phases of tuberculosis infection. Such approach may help in identifying new single antigens for serologic diagnosis of active tuberculosis.

Eur Respir J., 1993, 6, 816-822.

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Keywords: A60 antigen
antibody repertoire
tuberculosis

Received: June 11 1992
Accepted after revision March 24 1993

Active pulmonary tuberculosis (TB) is associated with strong antibody responses against the *Mycobacterium tuberculosis* (*M. tuberculosis*) [1, 2]. Work from several laboratories aimed at designing diagnostic serologic tests using purified proteins and specific monoclonal antibodies has shown great variability in serum antibody against *M. tuberculosis* in different TB patient groups [3-6]. Although HLA allelic background plays a role in the development of antibody responses against specific *M. tuberculosis* proteins in man as well as in the mouse [7-10], several studies have lent support to the concept that the intensity and the duration of infection, in addition to treatment, may modify the antibody response [8-10].

The *M. bovis* (BCG) antigen A60 is a complex of dominant antigens that induce a strong IgM and IgG-mediated immune response at the onset of active TB. The A60 complex has been used for the serological diagnosis of pulmonary, pleural and central nervous system TB [11-13]. In the context that strong reactions to A60 in inactive TB may hinder diagnostic specificity of A60-based immunoassays, we reasoned that the identification of antibody responses to specific components of the complex, might help with diagnosis of new cases with active TB.

To test this possibility, a mixed longitudinal study [14] was designed to prospectively analyze antibody responses to A60 proteins during *M. tuberculosis* infection. To encompass the time course of disease the study included purified protein derivative-negative (PPD⁻) and PPD⁺ normal individuals, and patients affected with TB. These were evaluated at the clinical onset, or 2-6 months after the initiation of chemotherapy or one year after treatment and resolution of active TB.

The data obtained demonstrate that during the course of pulmonary TB, specific antibody responses undergo changes that i) are indicative of a cell-maturation process involving Ig isotype usage switching and expansion of the repertoire against specific proteins of the A60 complex, and ii) may distinguish active TB-associated responses from memory-type responses.

Methods

Study Population

The study population (table 1) included normal PPD⁻ individuals as the non-infected control population, normal

PPD⁺ individuals as the *M. tuberculosis* - infected control population, TB patients at the onset of active disease [15], during chemotherapy, and after treatment and resolution of active disease.

Normal PPD⁻ controls ("PPD⁻"), were 4 males and 6 females (average age 27±2 yrs), all were Caucasian; all were PPD⁻, and none had history of TB or of *M. tuberculosis* exposure. Normal PPD⁺ exposed individuals ("PPD⁺"), were 7 males and 2 females (average age 33±3 yrs), all were Caucasian; all were currently exposed to *M. tuberculosis* as health workers, all were PPD⁺ and none had TB history.

Patients at the onset of active TB were 24 individuals without previous TB history, admitted to the hospital because of suspected active TB. They were 14 males and 10 females (average age 37±4 yrs); 17 were Caucasian, 2 Asian, and 5 northern African. All had positive *M. tuberculosis* cultures in Loewenstein-Jensen medium from sputum and/or bronchoalveolar lavage material. None of the patients in the study had associated human immunodeficiency virus (HIV) infection. All were evaluated before the beginning of chemotherapy. Thereafter they received standard anti-tuberculosis therapy including isoniazid (INH) (5 mg·kg⁻¹·day⁻¹), rifampicin (10 mg·kg⁻¹·day⁻¹), ethambutol (25 mg·kg⁻¹·day⁻¹), streptomycin (1 g·day⁻¹ up to 1 g·kg⁻¹). The onset active TB group was further stratified into two subgroups: an acid fast bacilli-positive (AFB⁺) culture-positive subgroup (n=10), who had positive AFB sputum smear at admission, and an acid fast bacilli-negative (AFB⁻) culture-positive subgroup (n=14).

Patients with active treated TB were 19 individuals with active TB, all with positive *M. tuberculosis* cultures at admission, which were evaluated two months after the initiation of specific chemotherapy. They were 14 males and 5 females (average age 45±3 yrs); 16 were Caucasian, 1 Asian, and 2 northern African. In addition to this group, a subset of 10 patients from the onset TB group were studied again two to six months after the initiation of treatment. This group was not statistically different from patients in the onset group for any of the demographic, clinical and immunological parameters. The active treated TB group was also stratified into two subgroups, AFB⁻/culture⁺ group (n=9) and AFB⁻/culture⁻ (n=10) group. No patients in this group were either AFB⁺/culture⁺ or AFB⁺/culture⁻.

Patients with resolved, past-active TB were 20 individuals who had completed treatment with resolution of TB, at least one year before the study. They were 9 males and 11 females (average age 38±5 yrs); 19 were Caucasian, and 1 northern African.

Patients with non tuberculous pulmonary granulomas were 15 individuals with biopsy proven pulmonary sarcoidosis. They were 6 males and 9 females (average age 35±4 yrs); all were Caucasian.

Nonspecific markers of disease activity

The activity of the immune processes during TB was gauged by quantifying serum levels of a molecule that is released by activated lymphocytes and macrophages [16].

Serum levels of neopterin (Neopterin, IBL) were measured by immunoassay according to the recommendations of the manufacturer.

Antibody levels

Serum levels of anti-*M. tuberculosis* IgM and IgG antibodies were quantified by immunoassay using A60 (a high molecular weight immunodominant antigen complex extracted from *M. bovis* BCG [11-13]) as the antigen. A commercial enzyme linked immunosorbent assay (ELISA) kit (TB-test, Eurospital-Pharma, Trieste, Italy) was used following the recommendations of the manufacturer. Serum antibody levels were transformed into normalized indexes, using a serum standard.

Antibody repertoire

Western blot analysis, with A60 as the antigen (a gift of C. Cocito, Microbiology and Genetics Units, University of Louvain, Brussels, Belgium), was used to evaluate anti-*M. tuberculosis* antibody specificities. Briefly, 300 µg of chromatographically purified A60 were fractionated on SDS polyacrylamide gel by electrophoresis under reducing conditions, and transferred onto nitrocellulose (NC) membranes (Biorad, Richmond, CA, USA). Membranes were cut in 4 mm wide strips, incubated with blocking buffer (50 mM Tris, 1% Tween 20, NaCl 150 mM (all products from Sigma, St. Louis, MO, USA)), and sequentially reacted with sera (1:2 dilution), anti-human immunoglobulin biotin-conjugated goat antibody (Sigma) and extravidin-conjugated peroxidase (Sigma). Antibody binding was revealed using 4Cl-1 naphthol (Sigma) with 0.03% H₂O₂ (Sigma). An anti-A60 rabbit antiserum, obtained by immunizing rabbits with A60 in Freund's incomplete adjuvant, which recognized more than 16 fractions on A60 blots and only 5 fractions on *E. Coli* blots, was used as a positive control. Molecular weight markers used were rabbit muscle phosphorylase b (97 kDa), bovine serum albumin (66 kDa), hen egg white ovalbumin (45 kDa), bovine carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), and hen egg white lysozyme (14.4 kDa) (Biorad).

Identification of specific proteins

Molecular identification of the A60 proteins bound by sera, was carried out by a western blot competition assay [17] using a panel of anti-*M. tuberculosis* monoclonal antibodies, that included antibodies TB78 and ML30 (both recognizing a 65 kDa heat shock protein (HSP) [3, 18]), TB72 and TB71 (both recognizing a 38 kDa *M. tuberculosis*-specific protein [19]), and TB68 (recognizing a 14 kDa *M. tuberculosis*-specific protein [20]), all generously provided by J. Ivanyi (Medical Research Council, Tuberculosis & Related Infections Unit, Royal Postgraduate Medical School, London, U.K.). Briefly, NC strips

were incubated with test sera (1:1, 1:5, and 1:25 dilutions) and then reacted (90% of optimal dilution) with monoclonal antibodies. Binding was revealed with a biotin-conjugated anti-mouse rabbit antibody (Sigma).

Analysis of anti-A60 serum antibody classes

Analysis of the antibody classes involved in the anti-*M. tuberculosis* response was carried out by IgG and IgM-specific western blot analysis. Anti-human IgG or IgM biotin-conjugated goat antibodies (Sigma) were used with extravidin peroxidase as above. A subset of 10 individuals from each study group was evaluated. Each of the subgroups evaluated for isotype usage did not differ from the corresponding study group for any of the immunological markers or antibody levels.

Statistical evaluation

Data are expressed as mean±standard error of the mean (SEM). Comparisons between groups were performed using one way variance analysis. Comparisons between percentages were performed using the χ^2 test.

Results

Immune activation during TB

Serum levels of neopterin were significantly elevated at the onset of TB ($p<0.001$, compared to normals (PPD⁺), table 1), irrelevant of the AFB⁺/⁻ status; they were still elevated 2 months after the beginning of anti-TB treatment ($p<0.003$ compared to normals (PPD⁺), table 1), while they were within the normal range after therapy and recovery ($p<0.002$ compared to onset TB, and $p>0.05$ compared to normals (PPD⁺), table 1). Neopterin levels in individuals with active sarcoidosis were markedly elevated (table 1).

The data indicate that the immune response to tuberculous infection was still strongly active in all patients treated with standard anti-mycobacterial therapy after two months, *i.e.* a time when constitutional symptoms had disappeared and sputum exam for AFB had already converted to negative.

Anti-A60 antibody level and repertoire of normal individuals

Analysis of the anti-tuberculous antibody response of PPD⁻ normal controls showed that, albeit in the presence of low antibody levels (table 1) they had anti-tuberculous antibodies against proteins of 67 to 63 and 62 to 57 kDa (fig. 1). In contrast, the anti-tuberculous antibody repertoire of PPD⁺ normal individuals, notwithstanding a similarly low serum antibody level (table 1), encompassed *M. tuberculosis*-specific proteins of 38 kDa (PPD⁻ 0%, PPD⁺ 88% responders; $p<0.005$), 32 to 30 (PPD⁻ 30% and PPD⁺ 88% responders, $p<0.03$), 29 to 28 (PPD⁻ 0% and PPD⁺ 88% responders, $p<0.005$), 25 to 22 (PPD⁻ 0% and PPD⁺ 66% responders, $p<0.02$) and, in some of them, 19 kDa (PPD⁻ 0% and PPD⁺ 45% responders, $p<0.05$), all antigens that were not recognized by PPD⁻ controls (fig. 1).

Competition western blot with PPD⁻ and PPD⁺ normal sera showed that antibodies directed against 67 to 63 kDa proteins recognized the immunodominant 65 kDa heat shock protein (HSP65). Serum antibodies from all these individuals were able to block the binding of both the TB78 (data not shown) and ML30 mouse monoclonal antibodies (both directed against *M. tuberculosis* HSP65; fig. 2A). In contrast, when mouse antibodies TB72 (fig. 2B) and TB71 (data not shown), that recognize a *M. tuberculosis*-specific 38 kDa protein, were tested in the same assay only the serum from PPD⁺, but not from PPD⁻ normals blocked monoclonal antibody binding.

Analysis of immunoglobulin isotype usage in the response against *M. tuberculosis* of PPD⁺ controls showed that these individuals recognized A60 complex dominant antigens of 65, 38, 32, 28, 25–22 and 19 kDa, with IgM as well as with IgG antibodies (table 2).

Table 1. - Evaluation of nonspecific immune activation intensity and of specific anti-A60 antibody levels in individuals with onset active tuberculosis, active treated tuberculosis and past active tuberculosis, and in immune and non-immune normals

	Patients n	AFB %	Serum Neopterin ng·ml ⁻¹	Serum Anti-A60 IgG*	Serum Anti-A60 IgM*
PPD ⁻ Normals	10	-	-	2.01±0.26 (0%)†	0.72±0.07 (0%)†
PPD ⁺ Normals	9	-	0.67±0.04	2.16±0.39 (22%)	0.78±0.09 (11%)
Onset Active Tuberculosis	24	58	1.11±0.07	23.5±5.36 (95%)	2.07±0.36 (65%)
Active Treated Tuberculosis	19	0	1.31±0.12	48.8±8.91 (94%)	1.78±0.31 (53%)
Past Active Tuberculosis	20	0	0.72±0.05	21.8±6.99 (84%)	0.73±0.12 (13%)
Sarcoidosis	15	0	3.21±0.31	2.11±0.12 (7%)	0.73±0.09 (7%)

Results are expressed as mean±SEM (normalized indexes) except for AFB. AFB: Acid Fast bacilli stained sputum smears (Ziehl Neelsen), percent positive at the time of evaluation; *: Serum anti-A60 antibody (normalized index). †: Percentage of individuals with abnormal antibody level (2sd above PPD⁻ control mean level). IgG, IgM: immunoglobulin G and M; PPD⁻/PPD⁺: purified protein derivative negative/positive.

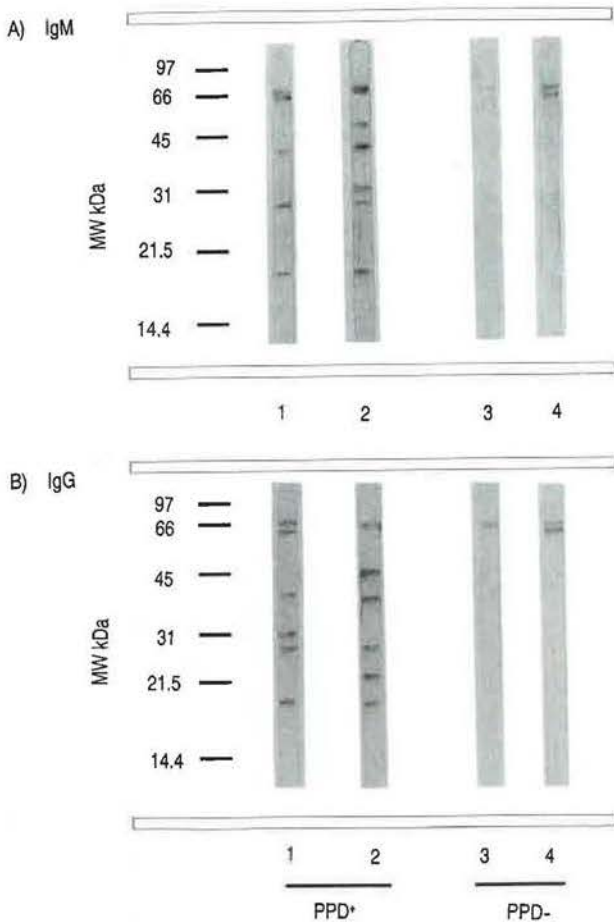


Fig. 1. - Western blot analysis of the antibody repertoire against *M. bovis* Antigen 60 (A60), of normal non-immune individuals (PPD⁻) and normal *M. tuberculosis*-exposed immune individuals (PPD⁺). Panel A: examples of IgM anti-A60 repertoire analysis of two normal PPD⁺ individuals (PPD⁺, lanes 1 and 2) and two normal PPD⁻ individuals (PPD⁻, lanes 3 and 4). Panel B: examples of IgG anti-A60 repertoire analysis of the same individuals (PPD⁺, lanes 1 and 2 and PPD⁻, lanes 3 and 4). MW: molecular weight; IgM and IgG: immunoglobulins M and G.

Anti-A60 antibody isotype usage during the time course of TB

Analysis of serum anti-A60 IgM levels in patients with pulmonary TB showed that a significant fraction of individuals at the onset of disease had elevated IgM levels ($p < 0.01$ compared to PPD⁺ controls; table 1). The AFB⁺ and AFB⁻ subgroups had similar specific IgM serum levels. A significant proportion of individuals with active TB during chemotherapy also had elevated IgM levels ($p < 0.01$ compared to PPD⁺ controls; table 1). Individuals with resolved TB had normal IgM antibody levels ($p > 0.20$ compared to PPD⁺ controls; table 1). In contrast, all individuals with TB, irrelevant of the phase of disease, had elevated anti-A60 IgG antibody levels (onset TB, $p < 0.01$; treated TB, $p < 0.01$; resolved post-treatment TB, $p < 0.01$; all compared to PPD⁺ controls; table 1). Interestingly, among individuals with onset active TB, those which were AFB⁻/culture⁺ *i.e.* those having less extensive disease, had higher IgG levels (32.1 ± 9.13), compared to the AFB⁺/culture⁺ subgroup (17.3 ± 5.58 , $p < 0.04$). Similarly, among treated active TB patients, those with negative sputum (AFB⁻/culture⁻) had higher IgG levels (70.0 ± 10.59), compared to individuals with persistent culture⁺ sputa (31.0 ± 10.53 , $p < 0.001$).

Although all TB patients had IgM as well as IgG antibodies against 67 to 63 kDa proteins, the antibody repertoire and isotype usage against TB-specific antigens showed significant changes during the course of disease (table 2).

Individuals studied at the clinical onset of pulmonary TB, had both IgM and IgG antibodies against the 38 kDa, a 32 kDa and a 28 kDa protein. In contrast, the antibody response against the same proteins in individuals treated with two months of chemotherapy was dominated by IgG antibodies (anti-38 kDa IgM vs. IgG $p < 0.03$; anti-32 kDa IgM vs. IgG $p < 0.05$; table 2). Further, the antibody response of individuals with resolved TB was almost exclusively IgG-mediated (anti-38 kDa IgM vs. IgG

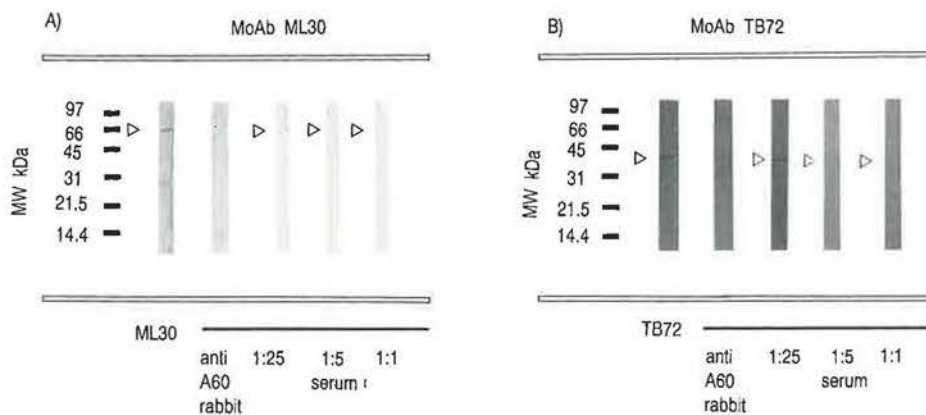


Fig. 2. - Identification of A60 protein species recognized by normal PPD⁺ individuals, using a western blot competition assay with anti-*M. tuberculosis* mouse monoclonal antibodies (MoAb). Panel A: antibody ML30 (directed against the HSP 65 *M. tuberculosis* protein, lane ML30) blocked by anti-A60 rabbit antibody (lane anti-A60 rabbit); and by increasing concentrations of normal PPD⁺ serum (lanes 1:25, 1:5, 1:1). Panel B: antibody TB72 (directed against the *M. tuberculosis*-specific 38 kDa, lane TB72) blocked by the anti-A60 rabbit antibody (lane anti-A60 rabbit); and by increasing concentrations of normal PPD⁺ serum (lanes 1:25, 1:5, 1:1). Molecular weight markers (MW (kDa) are shown to the left. \triangleright : Indicates the protein fraction recognized by specific antibodies. For abbreviations see legend to figure 1.

Table 2. - IgM and IgG isotype usage against A60 antigen complex specific proteins in patients with tuberculosis

MW kDa	100-68	67-63	62-58	57-53	52-50	49-46	45-44	43-41	40-39	38-36	35-33	32-30	29-28	27-26	25-22	21-14
IgM % responders																
PPD*Normals	0	100	50	0	0	0	0	0	0	0	0	20	0	0	0	0
PPD*Normals	45	100	100	0	45	0	0	0	0	88	0	88	77	0	66	45
OAT	10	100	70	0	40	30	40	10	0	60	0	20	70	0	20	0
AT	0	100	100	0	0	0	0	0	0	20	0	40	30	0	40	100
PAT	0	100	60	0	0	0	0	0	0	0	0	0	0	0	0	0
SARC	0	100	27	0	0	0	0	0	0	13	0	27	20	0	0	27
IgG % responders																
PPD*Normals	0	100	50	0	0	0	0	0	0	0	0	30	0	0	0	0
PPD*Normals	45	100	100	33	0	77	0	0	0	88	0	88	77	0	55	45
OAT	0	100	70	0	40	0	20	0	0	80	0	60	70	0	0	0
AT	20	100	100	0	0	0	0	0	0	80	0	90	60	0	40	20
PAT	30	100	60	0	20	0	20	0	0	100	20	60	70	0	60	100
SARC	0	100	27	0	0	0	0	0	0	13	0	27	13	0	0	7

IgM/G: Immunoglobulin M/G-specific western blot analysis; PPD*: normal controls (n=10); PPD+: normal controls (n=9); OAT: Onset active tuberculosis patients (n=10); AT: Active treated tuberculosis patients (n=10); PAT: Past-active tuberculosis patients (n=10); SARC: Sarcoidosis patients (n=15).

p<0.0005; anti-32 kDa IgM vs. IgG p<0.02; anti-28 kDa IgM vs. IgG p<0.01; table 2). Individuals that at the onset of TB had no antibodies against the 19 kDa protein, showed an IgM-dominated response after 2 months of treatment (p<0.005 compared to disease onset; table 2).

Analysis of sera from a group of individuals who were studied both at clinical presentation and 4-6 months after the initiation of chemotherapy showed that antibodies against 25-22 and 21-14 kDa *M. tuberculosis* proteins, which were not recognized at the onset of disease, appeared during the course of infection (fig. 3). Antibodies against 25-22 kDa proteins, that were observed in only 10% of these patients when first studied, were present in 60% of them after 4-6 months (p<0.02). Similarly, antibodies against the 19 kDa protein, that were observed in only 10% of patients at the onset of disease, were present in 50% of them after 4-6 months (p<0.02).

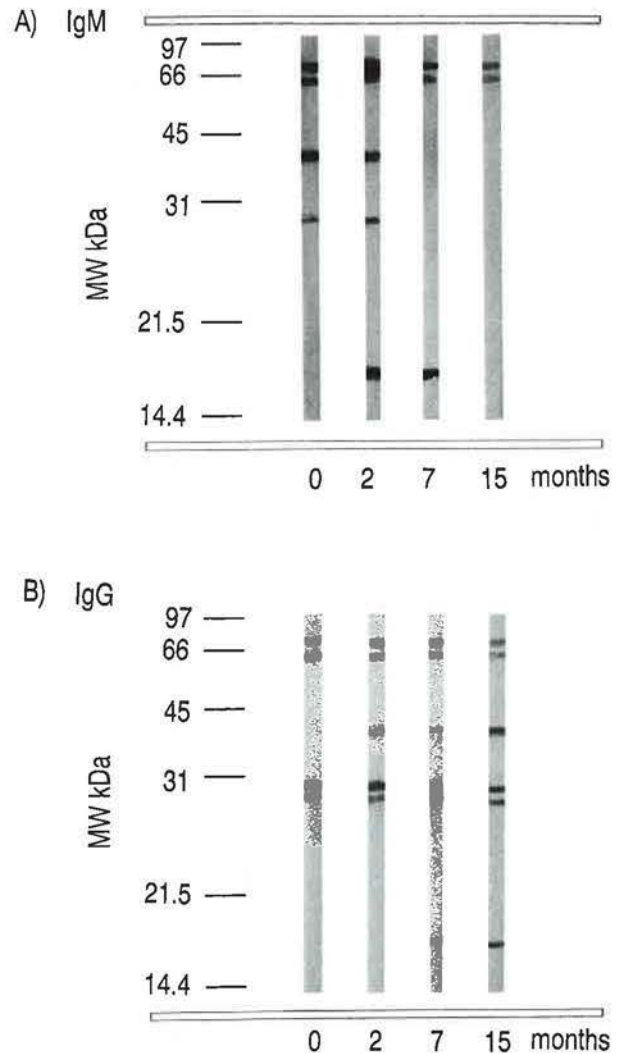


Fig. 3. - Western blot analysis of the anti-A60 antibody repertoire of patients with active TB. Shown are examples of sequential analysis of anti-A60 antibody repertoire of an individual treated for active TB; at the onset of disease (0), and after 2, 7 and 15 months. IgM anti-A60 patterns are shown in panel A; IgG anti-A60 patterns in panel B. Molecular weight markers (MW kDa) are shown to the left. For abbreviation see legend to figure 1.

The *M. tuberculosis*-specific 14 kDa protein (as determined by western blot competition with the antibody TB68) was not recognized by any of the patients at the onset of disease (data not shown). In contrast, it was recognized by 6% of chemotherapy-treated active patients, and by 30% of individuals with resolved TB ($p < 0.02$ compared to TB onset; $p < 0.05$ compared to active treated TB).

Anti-A60 antibody level and repertoire in sarcoidosis

The evaluation of patients with sarcoidosis, a non-tuberculous pulmonary granuloma, showed that both the levels (IgM 0.73 ± 0.09 , $p > 0.40$ compared to PPD⁻ normal control subjects; IgG 2.11 ± 0.12 , $p > 0.50$ compared to PPD⁻ normal control subjects) and the repertoire of anti-A60 antibodies were not different from PPD⁻ normal subjects. All these patients had antibodies against the 65 kDa HSP, and 27% had antibodies against the *M. tuberculosis* 32 kDa protein ($p > 0.50$, compared to PPD⁻ normal subjects). Although 4 out of 15 had antibodies against the 19 kDa protein, the responses to the 38, 28, 24 and 14 kDa *M. tuberculosis* proteins, were similar to PPD⁻ normal individuals.

Discussion

The analysis of antibody response and repertoire against *M. tuberculosis* using the antigen complex A60 as a probe showed that the early IgM antibody reaction against A60 dominant antigens develops into a broader IgG-mediated response during the course of active TB.

The antibody response of normal individuals against A60 clearly distinguished PPD⁺ from PPD⁻ individuals. Compared to PPD⁻, PPD⁺ normals had slightly higher anti-A60 levels and a markedly broader repertoire. PPD⁻ normals recognized the HSP65 and the HSP59 (data not shown). Both PPD⁺ and PPD⁻ also recognized proteins of 65 and 59 kDa on *E. coli* western blots (data not shown), thus suggesting that the antibody response against such proteins may not be specific for *M. tuberculosis* infection. In contrast, while only few PPD⁻ normals recognized a 32 to 30 kDa A60 protein, but not lower molecular weight fractions, PPD⁺ normals recognized four to eight fractions, including the *M. tuberculosis*-specific 38, and 19 kDa proteins. Interestingly, the antibody repertoire against A60 of PPD⁺ normals comprised low level antibody responses both of IgM and IgG isotypes. As for delayed type hypersensitivity [21–23], it is possible that latent infection foci are responsible for the maintenance of an active anti-*M. tuberculosis* IgM antibody response.

Individuals with clinically active infection presented with markedly increased production of the same IgM and IgG antibodies seen in PPD⁺ normals. Consistent with other studies [3, 4, 24], antibodies against the immunodominant 65 kDa HSP were present in all patients with active TB, in past-TB patients as well as in normals and in patients with sarcoidosis. In contrast, the antibody response against lower molecular weight *M. tuberculosis*-

specific antigens varied at different time points during the course of infection. Contrary to PPD⁺ normals, its development in TB patients was associated with markedly elevated antibody levels. Further, since it was not seen in pulmonary sarcoidosis patients, it is likely to be specific of *M. tuberculosis* infection.

As expected for an acute infection, at the clinical onset of TB the response against most high molecular weight (28 to 65 kDa) A60 fractions comprised both IgM and IgG antibodies. However, IgG antibodies against lower molecular weight A60 fractions appeared later in the course of disease, and were associated with more limited (AFB⁻) disease. In contrast, IgM antibodies against A60 lower molecular weight fractions disappeared following treatment with chemotherapy. Furthermore, in active TB patients, the IgM to IgG isotype switch occurred at a time when T-cell proliferation and gamma-interferon production against *M. tuberculosis* antigens by blood mononuclear cells are known to peak [25–27]. These observations lend support to the concept that the anti-A60 serum IgG antibody response may reflect the development of anti-*M. tuberculosis* cell-mediated immunity.

Several observations suggest that antibody responses follow stage-specific patterns. First, in experimental TB, the intensity of infection appears to play a role, together with genetic background, in determining the antibody repertoire [8–10]. Second, the antibody response against *M. tuberculosis* proteins such as the 32 kDa *M. bovis* protein [5], the *M. tuberculosis* MTP40 [6] and the *M. tuberculosis* 14 and 19 kDa proteins [3, 4], vary with disease intensity. Third, the antibody responses following immunization with certain low molecular weight mycobacterial antigens may take months to develop [28].

These findings are relevant to the design of serological tests for the diagnosis of TB. Serum immunoblotting analysis with the A60 antigen complex, which comprises several highly dominant, monoclonal antibody-identified, *M. tuberculosis* antigens, may be a powerful tool to dissect the anti-tuberculous immune response.

New molecular techniques and mouse monoclonal antibodies directed toward highly specific epitopes have been adapted to the serological diagnosis of TB [2–4]. The identification of antigens associated with antibody responses at the onset of active disease, may help with designing new specific assays aimed at detecting IgM antibodies [29]. In this context, further studies concerning the antibody response directed against the immunodominant T-cell epitopes expressed by A60 proteins of 21–24 and of 44–49 kDa, may identify new *M. tuberculosis* proteins of diagnostic value.

Acknowledgments: This work was supported by: "IV, V (ISS 6206-094) and VI (ISS 8205-23) Progetto di Ricerche sull'AIDS (1991-93)" Ministero della Sanità, Istituto Superiore di Sanità, Roma; by "Progetto Finalizzato FATMA", C.N.R. Italy; and by contract 90.02470.04, C.N.R., Italy. The authors thank D. Gueritore (University "La Sapienza", Roma) and G. Pallotta (Forlanini Hospital, Roma) for their invaluable support and hospitality; O. Pugliese (Istituto Superiore di Sanità Roma) for the gift of anti-A60 rabbit antibody; D. Giannarelli (Regina Elena Hospital, Roma) for help with statistical analysis and A. Amorese for secretarial work.

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