Eur J Pediatr (2003) 162: 534–536
DOI 10.1007/s00431-003-1199-6

RESEARCH LETTER

**Claudia Rolinck-Werninghaus · Klaus Magdorf
Klaus Stark · Konstantin Lyashchenko
Maria Laura Gennaro · Roberto Colangeli
T. Mark Doherty · Peter Andersen · Georg Plum
Udo Herz · Harald Renz · Ulrich Wahn**

The potential of recombinant antigens ESAT-6, MPT63 and mig for specific discrimination of *Mycobacterium tuberculosis* and *M. avium* infection

Received: 1 July 2002 / Accepted: 5 February 2003 / Published online: 25 April 2003

The tuberculin skin test is currently the only accepted immunological diagnostic tool available to detect infection with *Mycobacterium tuberculosis*. Immunological diagnosis of active tuberculosis (TB) in childhood is essential, since chest X-ray findings are less characteristic than in adults and as many as 60%–70% of cases remain culture-negative. However, tuberculin is not specific for *M. tuberculosis* due to the presence of epitopes shared with *M. bovis* BCG and non-tuberculous mycobacteria (NTM), such as *M. avium*. Since therapy of infections with *M. tuberculosis* and NTM differs, we assessed whether defined antigens for in vitro testing may improve diagnostic strategies.

Data from human studies have shown that one of these antigens, ESAT-6, elicits strong in vitro T-cell immune responses in adult TB patients but not in healthy tuberculin (PPD, purified protein derivative) skin test negative individuals (reviewed in [1]). MPT63 elicits specific humoral immune responses in humans with TB [3]. For the *M. avium* antigen mig specific antibody production in humans with *M. avium* infection (MAC) has been described [5]. Currently, there is only limited knowledge on the diagnostic potential of these antigens to discriminate between *M. tuberculosis* and MAC infection in T-cell-based tests in humans [2], and no data on paediatric mycobacterial infections are available.

In our experiments, PPD induced in vitro proliferation in 29/30 (Fig. 1A) and interferon-γ (IFNγ) secretion in 20/24 of TB patients, but these responses were not specific for *M. tuberculosis* since it was also recognised by 8/10 of MAC patients and 8/21 of healthy PPD skin test negative controls (IFNγ secretion in 2/9 and 0/20, respectively). Neither results in healthy skin test negative individuals nor in TB patients were influenced by BCG vaccination status. In contrast, ESAT-6 induced strong and highly specific in vitro proliferation in 20/27 and IFNγ-secretion in 11/24 of TB patients, but not in MAC patients or controls (Fig. 1B). The differences in responder rates of TB patients and controls or TB and MAC patients were highly significant. Both responders and non-responders to ESAT-6 showed comparable reactivity to PPD (Table 1).

MPT63 induced proliferation (Fig. 1C) and IFNγ release from peripheral blood mononuclear cells (PBMC) from a subgroup of TB patients (6/30 and 2/24, respectively) but not from those of MAC patients or controls (responder rates TB versus MAC, $P = 0.01$; TB versus controls, $P = 0.001$). One TB patient showed in vitro recognition of MPT63 but not of ESAT-6. The magnitude of proliferative responses induced by ESAT-6 in TB patients was significantly higher than that by MPT63 ($P < 0.001$), but was lower than that by PPD ($P < 0.001$, Fig. 1A–C). Comparable differences were

C. Rolinck-Werninghaus (✉) · K. Magdorf · U. Wahn
Department of Paediatric Pneumology and Immunology,
Charité, Humboldt University, Augustenburger Platz 1,
13353 Berlin, Germany
E-mail: claudia.rolinck-werninghaus@charite.de
Tel.: +49-30-450566182
Fax: +49-30-450566931

K. Stark
Robert-Koch-Institute, Berlin, Germany

K. Lyashchenko
Chembio Diagnostic Systems, Medford, USA

M. L. Gennaro
Public Health Research Institute, Newark, USA

R. Colangeli
Montefiore Medical Centre, New York, USA

T. M. Doherty · P. Andersen
Statens Seruminstitut, Copenhagen, Denmark

G. Plum
Institute of Medical Microbiology and Hygiene,
Cologne, Germany

U. Herz · H. Renz
Department of Clinical Chemistry and Molecular Diagnostics,
University of Marburg, Marburg, Germany

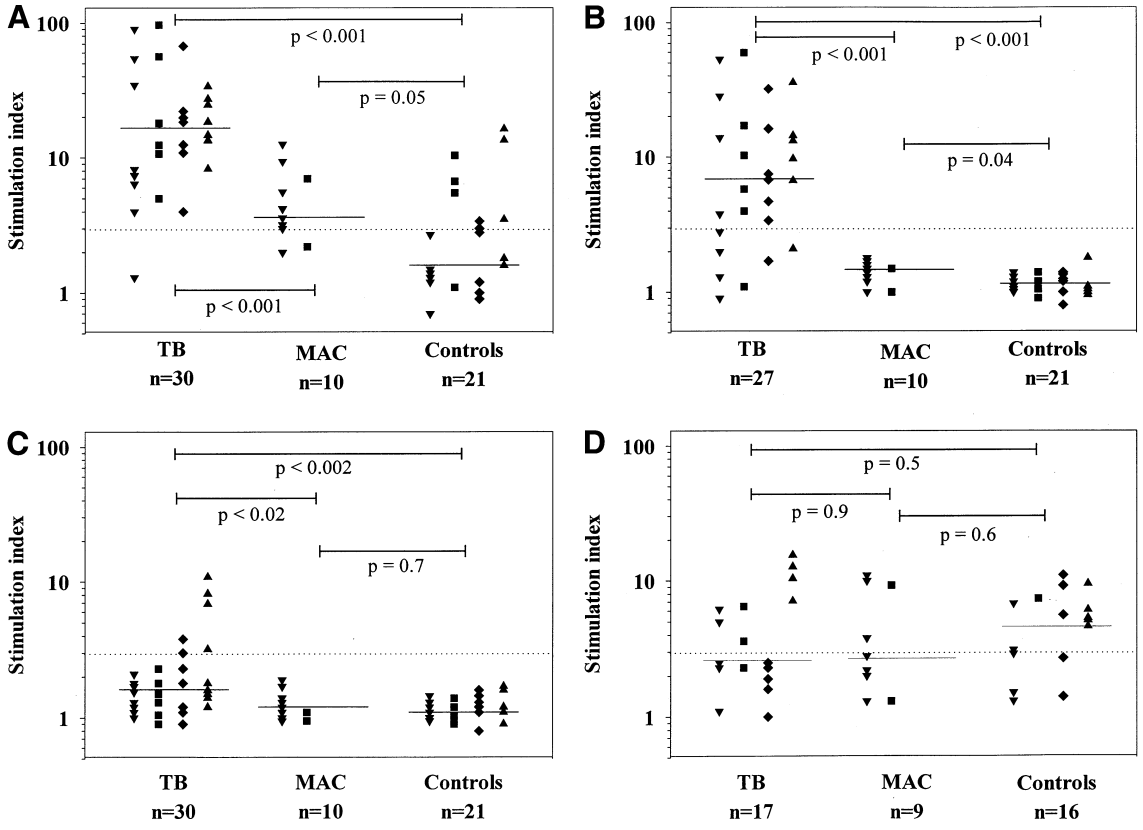

Fig. 1 Proliferation of PBMC from patients with TB, MAC or healthy, PPD skin test negative controls after stimulation with PPD or single recombinant antigens (age ≤ 16 years: without prior BCG vaccination *inverted solid triangles*, with vaccination *solid squares*; age > 16 years: without prior BCG vaccination *solid diamonds*, with vaccination *solid triangles*). **A** PPD (0.6 μg/ml). **B** ESAT-6 (1 + 5 μg/ml). **C** MPT63 (3 + 15 μg/ml). **D** mig (5 + 25 μg/ml). Purified PBMC were cultured for 5 days (37°C, 5% CO_2, positive control phytohaemagglutinin: 3 μg/ml for 3 days), pulse-labelled with 1 μCi ^{3}H-thymidine before scintillation counting. Stimulation index (SI; mean cpm of triplicate stimulated cells/non-stimulated cells) of maximal individual response to each antigen. SI ≥3: recognition assumed. *Horizontal bars* show means of respective results. IFNγ-secretion into culture supernatants was analysed by ELISA (IFNγ-OptEIA, Pharmingen, Germany). Cut-off for relevant IFNγ-secretion > 300 pg/ml

seen for analysis of IFNγ. The BCG vaccination status did not influence the responses to any of these antigens.

Results of mig-stimulated PBMC did not differ (Fig. 1D). In TB patients, but not in controls, PBMC from BCG vaccinated individuals proliferated with a higher frequency ($P = 0.006$), and the magnitude of responses was likewise higher (median stimulation index (SI) 13.7 versus 3.8, $P = 0.005$). Since the immuno-biological characterisation of mig has not been completed, a possibility remains that our data result from cross-reactive recognition, which is compatible with the increase in recognition in BCG vaccinated TB patients. The antigen preparation was free of LPS (Limulus assay).

Table 1 Characteristics of patients and healthy controls

		TB ≤ 16a	TB > 16a	MAC	Controls
n		14	16	10	21
Age [years, median (range)]		6.5 (1.7–15.1)	30.0 (16.4–39.9)	3.7 (1.5–8.5)	12.2 (0.9–44.2)
Abnormal chest x-ray		14	16	2	–
Culture (*n*)	positive	8	16	6	–
	negative	6[a]	0	4[b]	–
HIV negative		14	16	10	21
Skin test (*n*)	i.c. std. dose: < 10 mm	0	0	1	21
	i.c. std. dose: 10–14 mm	4	3	4	0
	i.c. std. dose: ≥15 mm	5	2	2	0
	i.c. low dose: > 10 mm	5	11	0	–

i.c. std. dose: equivalent to 5 TU PPD-S, i.c. low dose: equal to ≤ 0.5 TU PPD-S

[a]6/6 clinical response to antituberculous therapy; 2/6 recent smear positive TB exposition; further 2/6 positive *M. tuberculosis* complex PCR (twice). MAC patients: 8 cervical lymphadenitis with epithelioid cell granulomatosis, 2 pulmonary manifestation

[b]4/4 positive *M. avium* complex PCR (twice). HIV negative: serology negative + /CD4 > 25%/lymphoc. + > 1000/μl (< 6 years) or 500/μl (> 6 years)

Paediatric TB is predominantly a localised infection (hilar lymphadenopathy) and a paucibacillary disease, a potential cause for a different pattern of immune responses compared to adults. However, results for ESAT-6 in children (9/14) and adults (11/13) were comparable ($P = 0.4$). Differences were even less pronounced when only culture positive children were included (6/8, $P = 0.6$). Likewise PPD and mig had equal responder rates. However, MPT63 induced proliferation only in adult TB patients (6/16 versus 0/11 in children, $P = 0.02$). If only children with positive *M. tuberculosis* cultures were considered, then this was only a trend ($P = 0.07$) most likely due to lower patient numbers.

Our data show that in vitro stimulation of human PBMC with the secreted *M. tuberculosis* antigens ESAT-6 and MPT63 has a significantly higher potential than PPD for differentiating between infections with *M. tuberculosis* and with *M. avium*, the NTM species of highest relevance in humans. These could be used for the development of new, specific diagnostic tools. So far, only limited knowledge on its diagnostic potential to discriminate between these infections in humans has been available from eight adults with pulmonary *M. avium* disease and unknown HIV status [2]. In particular, ESAT-6 seems to be a potent candidate since antigen recognition between children and adults was comparable. We have further shown that T-cell recognition of mycobacterial antigens in children must be carefully assessed for the proper development of new diagnostic tools since it might differ from that in adults as seen for MPT63. While single antigens might not be sensitive enough for clinical diagnostic purposes, defined cocktails containing several specific antigens or peptides could be advantageous [4].

Acknowledgements We thank Gabi Schulz, Margret Oberreit and Petra Ellensohn for technical assistance and are grateful to Andreas Roth and Harald Mauch, Institute for Microbiology, Chest Hospital Heckeshorn, Berlin and the colleagues from Fachkliniken Wangen (Wangen), Johanniter-Hospital (Treuenbrietzen), St. Joseph's-Hospital, Children's Hospital Neukölln and Lindenhof (Berlin), Children's University Hospitals of Bochum and Frankfurt/Main, Altonaer Children's Hospital (Hamburg), Elisabeth-Hospital (Oldenburg), Children's Hospital (Wismar), and Horst-Schmidt-Hospital (Wiesbaden) for patient inclusion. This work was supported by the German Ministry of Education and Research (Grant No. FKZ-01KI9712).

References

1. Andersen P, Munk M, Pollok J, Doherty T (2000) Specific immune-based diagnosis of tuberculosis. Lancet 356: 1099–1104
2. Lein AD, von Reyn CF, Ravn P, Horsburgh CRJ, Alexander LN, Andersen P (1999) Cellular immune responses to ESAT-6 discriminate between patients with pulmonary disease due to *Mycobacterium avium* complex and those with pulmonary disease due to *Mycobacterium tuberculosis*. Clin Diagn Lab Immunol 6: 606–609
3. Lyashchenko K, Colangeli R, Houde M, Al Jahdali H, Menzies D, Gennaro ML (1998) Heterogeneous antibody responses in tuberculosis. Infect Immun 66: 3936–3940
4. Lyashchenko K, Manca C, Colangeli R, Heijbel A, Williams A, Gennaro ML (1998) Use of *Mycobacterium tuberculosis* complex-specific antigen cocktails for a skin test specific for tuberculosis. Infect Immun 66: 3606–3610
5. Plum G, Brenden M, Santos P, Schwarz E, Wahnschaffe U, Mauff G, Pulverer G (1996) Serum antibody reactivity to recombinant mig and whole cell antigens in *Mycobacterium avium* infection. Zbl Bakt 284: 348–360