ORIGINAL RESEARCH ARTICLE

Tanaffos (2004) 3(12), 7-18

Immune Reactivity of Mycobacterium Bovis (Strain BCG) and Mycobacterium Vaccae

Melani Rennou [1], Marie-C. Maes [1], H.H. Maes [1], R.F. Maes [1], Ali Reza Hadizadeh Tasbiti [2], Shamsi Yari [2], Ali Karimi [2], Shmasoldin Niknami [2], Ahmad Reza Bahrmand [2]

[1] Anda Biologicals, Strasbourg, FRANCE, [2] Pasteur Institute of IRAN

ABSTRACT

***Background**: The variability of the efficacy of M. bovis strain BCG used as a vaccine, and the controversial success of M.vaccae as an immunotherapeutic agent, lead the TB community to distrust these means to confront the resurgent TB problem. In addition, it is widely assumed that humoral antibodies against TB play only a minor role during mycobacterial infections.*

***Materials and Methods**: To shed some light on the reason for the observed failure of these immunological reagents, we analyzed the humoral immune response against the whole cells and the lysed cells of mycobacterial species M. tuberculosis, M.avium, M.paratuberculosis, M.vaccae and M.phlei, with antibodies raised against a pathogenic TB strain, against avirulent TB strain H37Ra, against A60 of BCG strain Pasteur GL-2 and against BCG strain Copenhagen. The reactivity of the thermostable macromolecular antigens (TMA) of BCG, M. paratuberculosis, M. phlei and M. vaccae, and of LAM extracted from BCG strain Pasteur GL-2 were also analyzed with these antibodies.*

The cellular immune activity of M. vaccae versus M. bovis strain BCG Pasteur GL-2 was analyzed by their capacity to induce an experimental arthritic syndrome.

Results and Conclusions**: Our results were that: **1-** the serological response obtained by whole cells and lysed cells of M. vaccae appeared most closely related to M. tuberculosis while that obtained by the cells and lysed cells of M .phlei was the most different. However, the antibodies directed against the antigen 60 complex of BCG strain Pasteur GL-2 did not react immunologically very strongly with the lysed cells and the TMA of M.vaccae, **2-** the antibodies against pathogenic TB cells, BCG sonicate strain Copenhagen and A60 from BCG Pasteur GL-2 recognized well whole cells and lysed cells of the BCG strains Copenhagen and Pasteur GL-2 but reacted poorly with the whole cells and lysed cells of a pathogenic TB strain and very poorly with the whole cells and lysed cells of BCG strain Aventis-Pasteur currently used as a vaccine (Monovax), **3-** the LAM extracted from BCG strain Pasteur GL-2 was poorly recognized by monoclonal antibody CS-40 directed against the LAM of Virulent TB strain Erdman but was recognized by monoclonal CS-35 antibody, that recognizes all LAMs. This LAM was recognized with the same efficacy by the four anti-mycobacterial antibodies used, including antibodies against the A 60 complex of BCG, **4-** the cell lysate and the TMA of M. vaccae did not stimulate as effectively the cellular immunity in vivo as did BCG extracts, as shown by their failure to induce an experimental arthritic syndrome under conditions that allowed its induction by the cell lysate and antigen 60 (TMA) of BCG.* ***(Tanaffos 2004; 3(12): 7-18)

***Key words:** BCG, M. Vaccae, Immunotherapy, Cellular Immunity, Humoral immunity, LAM*

Correspondence to: Bahrmand AR

Tel: +98-21-66968853, Fax: 098-21-66465132

Email address: padideh79@yahoo.com

INTRODUCTION

Interfron-γ, TNF-α and macrophage activation do not meet appropriately a virulent mycobacterial infection because these responses are activated with greater vigor by a virulent mycobacterial strain than by a low-virulent strain (1). This indicates that additional mechanisms of immunological resistance are needed to successfully meet an infection, namely humoral antibodies (2,3). We here expose our investigations on the immunological reactivity of M. vaccae and M. bovis BCG strains Copenhagen, Pasteur GL-2 and Aventis-Pasteur (Monovax Vaccine). Because various constituents of the bacilli are known to produce distinct responses (4,5,6,7,8), we analyzed whole cells, lysed cells, and Thermostable Macromolecular Antigens (TMA) (9) from various species. Antibodies against low molecular weight non-peptidic antigens are associated with ulterior development of TB disease (10), hence presumably not protective and not investigated in this study, but antibodies against LAM are thought to limit dissemination in childhood TB (11), and LAM of BCG was included in our study.

The host's cellular immune response is known to be an important element to combat infection. We evaluated the cellular immune activity of extracts of M. vaccae versus similar extracts of M. bovis strain BCG Pasteur GL-2 by assessing the capacity of these two strains to induce an experimental arthritic syndrome in rats (12). We are aware that this method of evaluation of the cellular immune response is not commonly used by the mycobacterial community. The advantage of arthritis over DTH for the evaluation of in vivo immune activity is that arthritic nubile women benefit from a considerable improvement of the disease during pregnancy, going as far as disappearance of symptoms, which resume after delivery. Arthritis appears thus to be a purely cellular immune reaction. Experimentally, it is induced in rats by the inoculation of a killed avirulent H37Ra strain and is a phenomenon that may be measured directly, without the intermediary of a challenging sensitin.

MATERIALS AND METHODS

1. Flow- through system for antigen detection

The interaction of antibodies with mycobacterial antigens was analyzed by the flow-through method, which has been already described in great detail (13). The main steps of the flow- through method are: the bacilli or cell components present in a liquid sample are used to sensitize a nitrocellulose membrane (Advanced microdevices, Ambala, cannt, India). The bacteria or cell components retained at the surface of the membrane are labeled with different rabbit antibodies raised against mycobacterial cells or cell components. The antibodies attached to the antigens are revealed with protein A-Gold conjugate (40 nanometers, British Biocell, UK). The intensity of the red coloration, indicative of the presence of rabbit gammaglobulins on the membrane, are visually estimated with a gradation extending from "-", i.e. absence of coloration, to "++++", indicative of intense coloration. Intermediate differences in intensity were noted by a sign "+", the lower limit of sensitivity being marked by the sign "+/-".

2. Antigens.

Antigens tested by this flow-through method consisted in whole bacterial cells, lysed cells from different bacilli, TMA from different mycobacterial species (including A 60 complex of BCG), dissolved TMAs and Lipoarabinomannan (LAM) from BCG strain Pasteur GL-2.

a) Bacterial cells:

1. Live biomass of M. bovis BCG bacilli, strain Pasteur GL-2, was a gift pf prof. Cocito (Faculty of Medicine, University of Louvain, Belgium).

2. Freeze-dried live M. bovis BCG bacilli,

Aventis-Pasteur vaccine Monovax lot W5586-2.

3. Live cultured pathogenic M. tuberculosis bacilli, courtesy of Dr. Bahrmand, Pasteur Institute of Iran, Tehran.

4. Biomasses of M. phlei, M. paratuberculosis and M. avium were from the National Veterinary Institute, Oslo, courtesy of Dr F. Saxegaard.

5. Biomass of M. vaccae, courtesy of Dr. Stanford, Middlesex school of Medicine, London.

The concentration of bacterial cells used for the determination of the specificity of antibodies was adjusted to 1 McFarland turbidity (3×10^8 cells/ml) and 50 µl, corresponding to 1.5×10^7 bacilli, were used in the tests.

b) The cytoplasm of the various bacilli was liberated from the cells by dissolving 50 µl of a suspension of bacterial cells adjusted at 0.2 McFarland turbidity during 15 minutes at room temperature in a solution of guanidine and detergents known to lyse the cells without destroying the immunological reactivity of the mycobacterial (13). This total cell lysate was passed on the flow-through device.

c) The thermostable Macromolecular Antigens (TMA) were obtained by cell disruption and chromatographic isolation by standard methods (14). The sensitization of the nitrocellulose membrane with Thermostable Macromolecular Antigens (TMA) was done by spotting 1 microgram of proteins dissolved in physiological saline on the membrane. The TMAs used to sensitize the membrane were:

1. Antigen 60 complex (A 60) of M. bovis, strain BCG Pasteur GL-2,

2. TMA of M. paratuberculosis (A 36),

3. TMA of M. phlei,

4. TMA of M. vaccae.

d) A fourth group of analyzed antigen was the above TMAs dissolved by a treatment identical to the one applied to lyse whole cells. The dissolution of the TMA complex by chaotropic agents and detergents in its primary components is achieved without destroying the immunological characteristics of its subunits (15). One microgram of dissolved TMA was used on each flow-through device.

e) Lipoarabinomannan was extracted from M. bovis BCG cells (strain Pasteur GL-2) according to known procedures (16). Briefly the cells were lysed in a French press; the lysate was clarified by centrifugation during 10 minutes at 15000 rpm and the supernatant extracted by phenol, followed by chloroform/ methanol the organic phase was then processed further by chromatography on Sephacryl S-100.

The LAM preparation contained 16 µg/ml protein, evaluated by the Bradford method using bovine serum albumin as a standard, and 227 µg/ml polysaccharides evaluated by the anthrone method using glucose as a standard. Again, one microgram in physiological saline was used for each flow- through device.

3. Antibodies

Antibodies originated from three different sources:

a) Polyclonal antibodies were produced by Neosystem, Strasbourg, France:

1. Rabbit antibodies against inactivated freeze-dried avirulent M. tuberculosis strain H37Ra (Difco 231141)

2. Rabbit antibodies against A 60 of M. bovis BCG-Pasteur GL-2

3. Rabbit antibodies against whole heat-inactivated pathogenic M. tuberculosis bacilli obtained from the Pasteur Institute of Iran.

Immunizing solutions for the production of antibodies by Neosystem were provided by the authors. They consisted of 3.75×10^8 heat inactivated pathogenic TB cells in 500 µl of physiological solution and of 450 µg inactivated M. tuberculosis H37Ra freeze-dried cells in 500 µl physiological solution. The A60 complex was a suspension of 1

mg/ml in buffered saline. The immunizing suspensions were homogenized with an equal amount of incomplete Freund adjuvant (IFA) and inoculated monthly in rabbits.

The antisera were precipitated with ammonium sulfate, dissolved in a tenth volume phosphate-buffered saline, dialyzed and clarified by low speed centrifugation.

b) Rabbit antibodies against a whole sonicate of M.bovis strain BCG Copenhagen were purchased from Dako, Denmark.

c) Antibodies against LAM were obtained from the Colorado State University, under contract NIH, NIAID No1 AI-75320 for Tuberculosis Research Materials and Vaccine Testing. Monoclonal antibody CS-35 is a mouse IgG3 directed against all LAMs. Monoclonal antibody CS-40 is a mouse IgG1 directed against TB strain Erdman LAM.

Reactivity of LAM with mouse monoclonals was evaluated by Dot-Blot. The LAM sample (5-7 µl) was used to sensitize a nitrocellulose membrane. After a wash during 1 hour at room temperature with 15 ml of a 0.5% casein solution, the membrane was contacted overnight at room temperature under agitation with 15 ml of an antibody solution suitably diluted in 0.5% casein. After three successive washes during 10 minutes each, the membrane was incubated during 2 hours with 15 ml of a solution of biotinilated anti-mouse IgG antibodies in 0.5% casein. After a thorough wash, these antibodies were revealed with avidine-peroxidase in bovine serum albumin at pH 7.2, and 4-chloro-naphtol.

Reactivity of LAM with polyclonal rabbit antibodies against mycobacterial cells and subunits was analyzed by the flow-through method, using one microgram LAM in physiological solution per flow-through device.

Contrary to what was done in the previous communication, where antibody dilutions were adjusted to obtain a significant immunological response with their homologous antigens diluted in sputum. In this study the antigens were suitably diluted in 0.9% NaCl and antibodies were applied at their maximum allowed concentration before the occurrence of unspecific absorptions.

4. Experimental arthritis syndrome.

The syndrome consists of the swelling of the hind-paws of rats. The syndrome is normally induced with a water-in-oil emulsion of Freund complete adjuvant (FCA) consisting in equal volumes of 85% mineral oil 15% arlacel and an aqueous suspension of 5 mg inactivated avirulent M. tuberculosis H37Ra strain, of which 50 µl are inoculated at the base of the tail of rats (12). The Sprague- Dawlay strain of rats is the most susceptible to this induced arthritic syndrome, which appears 8-10 days after inoculation (17).

In this study, incomplete Freund adjuvant (IFA), i.e. the oil and arlacel solution without the H37Ra extract, was vortexed with an equal amount of aqueous solutions containing various mycobacterial extracts to obtain a stable water-in-oil emulsion which was inoculated deep at the base of the tail of Sprague-Dawley rats. The protein concentration of the mycobacterial products was evaluated by the lowry method. All preparations were diluted in PBS to 800 µg protein/ ml before their incorporation in the emulsion.

The substances inoculated were IFA with:

1. Lysates of M. vaccae and M. bovis strain BCG Pasteur GL-2. These were obtained by destruction of the bacteria in a French press,

2. The thermostable macromolecular antigens (TMA) of M. vaccae and M. bovis

3. Physiological saline alone as a water in oil emulsion, and IFA with 800 µg/ml bovine serum albumin (two controls).

Evaluation of the swelling of the hind-paws of rats was done at regular intervals, starting from the first day following the inoculation up to 23 days

thereafter. The intensity of the swelling was rated by two independent observers from "-" (no sign of swelling) to "+" significant Swelling) and "++" (excessive swelling), and the subjectivity of their rating corrected by its matching with the various controls included in the experimental design.

RESULTS

1. Humoral immunity

Antibodies against 1) a pathogenic TB strain, 2) avirulent H37Ra TB strain, 3) BCG strain Copenhagen and 4) A60 complex of BCG Pasteur GL-2 were reacted with antigen originating from different mycobacterial species. We subdivided our serological results in five subsets of antigens: a) whole cells b) lysed cells c) TMA's d) BCG strains and e) whole cells b) lysed cells c) TAM's d) BCG strains and e) LAM.

a) Specificity of antibodies for the whole cells of different mycobacterial species.

The reaction between whole cells of 5 different mycobaterial species and four antimycobacterial antibodies indicate (Table 1) that the four antibodies subdivide into two groups. The antibodies against A60 complex of BCG strain Pasteur GL-2 form a group by themselves. These antibodies react moderately (score ++) against the whole of M. tuberculosis and M. vaccae, and poorly (score -/+ to +) against the whole cells of M. phlei, M. avium and M. paratuberculosis.

The second group of antibodies are those against the pathogenic TB strain, the avirulent H37Ra TB strain and the sonicate of BCG strain Copenhagen. These antibodies reacted well with whole cells of M. tuberculosis (score +++/- and +++) and very well (score ++++) with cells of M. vaccae. Their reactivity with whole cells of M. phlei, M. paratuberculosis and M. avium was globally reduced but better than their recognition by anti-A60 antibody.

Among them, reactivity against M. phlei cells appeared the weakest while reactivity with M. paratuberculosis cells appeared closest to M. tuberculosis. The intensity of the responses observed with M. avium antigens was consistently a degree lower than that obtained with M. paratuberculosis antigens. These two species are known to be immunologically very closely related (18). The weaker response against M. avium antigens was traced by us to aggregation of the capture antigen, due to the difficulty encountered in its homogenization. The discrepancy between the response of these two species waned when the cells were dissolved with a chaotropic agent, as shown infra.

Table 1. Interaction between 4 anti-mycobacterial antibodies and the whole cells and dissolved cells of 5 different mycobacterial species. Note that antibodies' concentration is adjusted to clearly distinguish weak from strong interaction during this particular experiment.

	Whole cells (1 McFarland: 50 µl)				
Antibodies against:	Pathog.TB	M. vaccae	M. phlei	M. paratub	M. avium
A60- BCG*	++	++	+/-	++/-	+
BCG ** sonicate	+++	++++	+/-	+++/-	+
Pathog. TB	+++/-	++++	+/-	++	++
H37Ra	+++	++++	++	++/-	+
	Dissolved whole cells (0.2 McFarland: 50 µl)				
A60-BCG *	+++	+	+/-	++/-	+
BCG**	+++	+++	+/-	+++	++
Sonicate	++++	+++++/-	++	++++	++++
Pathog. TB H37Ra	++++	++++	+++	++++	++++

*: Against A60 from BCG strain Pasteur GL-2

**: Against strain Copenhagen

This difference in serological reactivity of whole cells allowed to subdivide the five mycobacterial

species analyzed into two groups based on the strength of cell surface antigen recognition, the first being composed of M. tuberculosis and M. vaccae, the second of M. phlei, M. paratuberculosis and M. avium. Note that antibodies against pathogenic TB recognized better M. vaccae cells than TB cells.

b) Specificity of the antibodies for the lysed cells of mycobacterial species.

The cytoplasm was released from the cells by dissolving them into a solution of guanidine and detergents. The treatment amplified the immunological recognition of the antigens, and dissolved cells needed to be diluted fivefold before their application on nitrocellulose membranes.

The restricted specificity of the antibody against A60 complex from BCG strain Pasteur GL-2 was increased and only the dissolved cells of TB remained well recognized by this antibody (Table 1). The spectrum of reactivity of the antibodies against TB pathogenic strain and avirulent TB strain H37Ra was confirmed for pathogenic TB and vaccae lysates and extended to M. paratuberculosis and M.avium which separated from less recognized M. phlei. Note that lysate of M. phlei was still better recognized than whole cells, although diluted 5 fold. The serological similarity of M. paratuberculosis and M. avium (18) was confirmed. The spectrum of recognition of antibodies against a sonicate of BCG strain Copenhagen was not changed: it barely recognized M.phlei lysed cells.

c) Specificity of the antibodies for the TMA of mycobacterial species.

Table 2 indicates that the antibody against A60 from BCG (Pasteur GL-2) recognized best A60 from BCG (Pasteur GL-2), against which they were raised. Anti-A60 antibodies also recognized, although less efficaciously, the TMA of M. paratuberculosis, but reacted poorly with the TMA of M. vaccae and very poorly with the TMA of M. phlei.

The three other antibodies had a broader spectrum of recognition. They reacted well with the TMAs of M. bovis (BCG) and M. vaccae. The TMA from M. paratuberculosis was also well recognized by anti-pathogenic TB and anti-BCG (Copenhagen) antibodies but not by the antibodies against TB strain H37Ra. Note that M. phlei again separated immunologically from the other species, in that its TMA was poorly recognized by all antibodies used.

The dissolution of the TMAs in chaotropic agents unmasked epitopes hidden in the four native TMA complexes. Recognition of the TMAs by the two antibodies against TB (pathogenic strain and avirulent strain H37Ra) generally increased (table 2).

Table 2. Interaction between 4 anti-mycobacterial antibodies and the thermostable macromolecular antigens, and dissolved TMAs, of 4 different mycobacterial species. Note that antibodies' concentration is adjusted to clearly distinguish weak from strong interactions during this particular experiment.

	Thermostable Macromolecular Antigen (1 μg)			
Antibodies against:	BCG °	M. vaccae	M. phlei	M. parat. °°
A60- BCG*	++++	+	+/-	++
BCG ** sonicate	++++	+++	+	+++
Pathog. TB	+++	++++	++/-	+++
H37Ra	++++	+++	+	+/-
	Dissol. Thermost Macromolec. Antigen (1 μg)			
A60-BCG *	++++	++/-	++	++
BCG** sonicte	++++	+++	++	+++
Path. TB strain	++++	++++	++++	++++
H37Ra	++++	++++	++++	++++

°: From BCG strain Pasteur GL-2

°°: A36

*: Against A60 from BCG strain Pasteur GL-2

**: Against strain Copenhagen

However, their recognition by antibodies against

A60 from BCG (Pasteur GL-2) of BCG sonicate (Copenhagen) was not improved. In particular, M. phlei remained less well recognized by these two antibodies (score ++). The dissolved TMA from M. vaccae remained poorly recognized by antibodies against A60 from BCG (score ++/-).

In conclusion, M. vaccae presents epitopes on its cell surface that are better recognized by antibodies against a pathogenic TB strain (score ++++) than are the pathogenic TB antigens themselves (score +++/-), while on the other hand its TMA is serologically very different from A60 from BCG strain Pasteur GL-2.

Aware of the considerable immunological difference that may occur is surface antigens of pathogenic TB strains (13), we now wanted to verify if the same drifts could be observed among BCG strain and tested the reactivity of two anti-BCG antibodies against three different strains of BCG, using anti-TB antibodies and a pathogenic TB strain as internal references.

d) Specificity of antibodies for three different BCG strains.

Table 3 indicates that the antibodies against a pathogenic TB strain recognized better the cells of M. bovis BCG strains Pasteur GL-2 (score ++++) and Copenhagen (score +++++/-) than that of the cells of the pathogenic TB strain used for immunization (score +++). Reactivity against BCG vaccine Monovax from Aventis- Pasteur was the weakest (score ++).

The same gradation held for antibodies against A60 from BCG Pasteur GL-2. These antibodies recognized best Pasteur GL-2 cells (score ++++). The pathogenic TB strain was less well recognized (score ++) while the cells of Aventis-Pasteur Monovax were poorly recognized (score +).

The antibodies against a sonicate of BCG (Copenhagen) recognized best (score ++++) the cells of BCG strain Copenhagen, against which they were raised. The BCG strain Pasteur GL-2 was also well recognized (score +++), while the Pasteur (Monovax) strain used by Aventis and the pathogenic TB strain were less well recognized (score +++/- and ++). Dissolution of the cells in a chaotropic agent and detergents did not potentiate the recognition of the pathogenic TB antigens, indicating that there were no hidden epitopes unmasked by the treatment (table 3).

Table 3. Comparison of the interaction of 3 anti-mycobacterial antibodies with the whole cells (50 µl, 1 McFarland) and the dissolved cells of a pathogenic TB strain and 3 BCG strains (50 µl, 0.2 Mc Farland). Note that antibodies' concentration is adjusted to clearly distinguish weak from strong interaction during this particular experiment.

Antibodies to:	TB Pathog. Strain	BCG		
		Pasteur	Copenhagen	Aventis
A60- BCG *	++	++++	+++	+
BCG ** sonicate	++	+++	++++	+++/-
Path.TB	+++	++++	+++++/-	++
	Dissol. TB cells	Dissolved BCG cells		
A60- BCG*	+	++	++	+/-
BCG** sonicate	++/-	+++/-	+++	+
Path.TB	++	++++	++++	+

*: Against A60 from BCG strain Pasteur GL-2

**: Against strain Copenhagen

Recognition of the lysed cells by the three antibodies was generally worsened, with the exception of recognition of the BCG strain Pasteur GL-2 and Copenhagen by anti-pathogenic TB antibocies, which is a paradox. The lysed cells of Aventis-Pasteur (Monovax vaccine) reacted with the antibodies in a poorer way than did the lysed cells of the pathogenic strain and in a much poorer way than the lysed cells of the two other BCG strains. From

this, we retain that the BCG strains Copenhagen and Pasteur GL-2 approach the level of M. Vaccae when cell surface antigens are considered. The antigens of the outer cell membrane of the BCG Monovax vaccine appear different from those of BCG strains Pasteur GL-2 and Copenhagen and of the pathogenic TB strain.

e) Reactivity with LAM extracted from BCG strain Pasteur GL-2.

LAM extracted from the BCG strain Pasteur GL-2 reacted very poorly with monoclonal antibody CS-40 against LAM from TB strain Erdman but reacted with monoclonal antibody CS-35, which is an antibody against all LAMs. The LAM was recognized with the same intensity by all four antimycobacterial antibodies, including antibodies against A60 complex from M. bovis BCG strain Pasteur GL-2.

2. Cellular immune activity

The lysates of M. vaccae and M. bovis, strain BCG-Pasteur GL-2, as well as their TMAs, were emulsified in IFA and inoculated deep at the base of the tails of two rats. Controls using IFA emulsion alone and IFA plus bovine serum albumin were included. The amount of inducers used (800 µg/ml protein) was 6 times lower than the amount commonly used to induce the syndrome with certainty (5 mg of TB strain H37 Ra/ml) because our goal was not to treat an established syndrome with immunosuppressive substances (17) but to evaluate the induction potency of different mycobacterial extracts.

The vast majority of the results, recorded from day 1 to day 23 of the experiment, were negative and data are therefore not shown. The hind paws of all the inoculated rats remained unchanged until 8 days after inoculation. At later times, IFA emulsion alone, IFA plus BSA, IFA plus the lysate of M. vaccae cells and IFA plus the lysate of M. vaccae cells and IFA plus the TAM of M. vaccae failed to induce a change in paw thickness. The hind paws swelled considerably on day 9 to 11 for one of the two animals inoculated with IFA plus the lysate of M. bovis cells strain BCG Pasteur GL-2. The excessive swollen state of the paws regressed slightly thereafter to remain in a visible swollen state from days 14 to 23. The swelling induced with IFA plus A60 from BCG (Pasteur GL-2) followed exactly the same pattern indicating that the two BCG inducers possessed about the same potency, superior to that of the two homologous M. vaccae extracts. We did not attempt to evaluate the differences in a quantitative way.

DISCUSSION

In this investigation, the primary antibodies were used at their maximal concentration and the sensitizing antigens were diluted in physiological saline to obtain an adequate reactivity in a flow-through system. Under these experimental conditions, the humoral immune reactivity of antibodies against BCG strains Copenhagen and Pasteur GL-2, against A60 complex of BCG and against avirulent TB strain H37Ra with their respective homologous antigens was excellent. Whereas this correlation validates the results obtained by this investigative method, it also forces a critical examination of the discrepant cases unveiled in this study.

1. General immunological characteristics of mycobacterial species

1. The humoral immune reactivities of M. bovis and M. vaccae with TB are much superior to those displayed by M. phlei, M. paratuberculosis and M. avium. M. phlei stands apart from the other mycobacteria.

2. Whole cells, lysates and TMAs have different immunological reactivities toward antibodies,

3. Antigenic masking is sometimes observable

with the TMAs

4. The cellular immune response elicited with M. vaccae extracts in an experimental arthritis syndrome is weaker than the response elicited by M. bovis extracts analyzed under similar assay conditions.

2. BCG

1. Antibodies against whole pathogenic TB cells recognize BCG cells of strains Copenhagen and Pasture GL-2 better (score ++++) than homologous TB cells (score +++) and recognize these homologous TB cells surprisingly better than they recognize BCG cells of strain Aventis-Pasteur (score ++),

2. Antibodies against the cytoplasm of BCG strain Copenhagen recognize the cytoplasm of BCG Copenhagen (score ++++) but do not recognize very well whole pathogenic TB cell (++) nor their cytoplasm (score ++/-). Their still poorer reactivity with the cytoplasm of BCG Aventis (score +) was unexpected.

3. The immunological reactivity of the BCG-Pasteur GL-2 differs from the activity of the same strain (Monovax) used to make the vaccine Aventis-Pasteur. Different biological activities among batches of the same BCG strain have been already reported (19).

4. The cell lysate and the TMA of the BCG strain Pasteur GL-2 induce a cellular immune response in vivo evidenced by the arthritic syndrome. This observation confirms studies made in vitro (20) with activated lymphocytes.

Our data indicate that; compared to other mycobacterial species, the choice of M. bovis, strain BCG, as a vaccine was good. Exception is taken with the vaccine Aventis- Pasteur, which possesses some immunological characteristics similar to those observed with M. phlei. It is generally admitted that BCG prevents secondary proliferation but not primary TB infections, and is known to be variable according to vaccine strain. Our results corroborate this limitation. They suggest that the BCG not elicit the synthesis of antibodies able to interfere efficiently with the surface of pathogenic cells during the primary TB infection nor with the secretion of cytoplasmic mycobacterial immunosuppressors (21, 22), but elicits a cellular immune response able to prevent proliferation. In addition, the outer cell membrane of BCG presents a phenotypic variation according to strain. The efficacy of some BCG strains to synthesize antibodies against surface antigens of some pathogenic strains would therewith be further compromised.

3. LAM

LAM has been suspected to TB within macrophages (23). Antibodies against LAM presumably limit dissemination of TB (11, 24, 25). The creation of antibodies against LAM for protection against TB seems desirable. The four antibodies we created, against whole virulent TB, against BCG cytoplasm, against A60 and against avirulent TB H37Ra recognized LAM.

4. M. vaccae

1. M. vaccae whole cells react better with antibodies against a pathogenic TB strain (score ++++) than do the homologous pathogenic TB cells (scores +++ and less).

2. M. vaccae lysed cells react with antibodies against a virulent TB strain, and with less efficiency with antibodies against BCG strain Copenhagen.

3. M. vaccae TMA (homologous to A60 of BCG) reacts only very weakly with antibodies against A60 from BCG strain Pasteur GL-2 (score +). On this criterion, it is farther distant from the A60 of BCG (score ++++) than are the TMAs of M. paratuberculosis and M. avium (score ++),

4. M. vaccae lysed cells and TMA do not readily solicit a cellular immune reactivity in vivo, as shown

by their incapacity to induce experimental arthritis in rates under conditions where similar BCG products did. We do not here imply that M. vaccae is inert at that level but well that it is less efficient than killed BCG or A60 of BCG.

Compared to other mycobacterial species, the choice of M. vaccae for immunotherapy appears judicious. The evidence we have is that M. vaccae induces a desirable humoral response against cell surface antigens and cytoplasmic antigens but not against A60 antigen, and its capacity to mobilize the cellular immunity is weaker than that of BCG. These limitations restrict its effectiveness.

If immunological responses observed in rabbits are transposable to humans, one would be entitled to conclude that the application of the M. vaccae immunotherapy at the beginning of the chemo-treatment of immuno-depressed patients, make full use of the desirable immunological properties of M. vaccae. However, immunocompetent patients whose chemotratment is sufficient to heal the patients will, in this context, not benefit from the immuno-treatment because these patients are able to synthesize spontaneously the antibodies needed to fight the infection (26).

In the clinical situation of generalized immunosuppression in chronic cases, when all antigens required to mount a therapeutic immune response are available but unable to trigger it despite the chemical help, the inability of M. vaccae to elicit a cellular immune response in vivo equal to that of the BCG is a handicap that would be overcome only by repeat inoculations of the immunotherapeutical (27). An alternative would be the incorporation of A60 into the immunotherapeutic.

REFERENCES

1. Florido M, Goncalves AS, Silva RA, Ehlers S, Cooper AM, Appelberg R. Resistance of virulent Mycobacterium avium to gamma interferon-mediated antimicrobial activity suggests additional signals for induction of mycobacteriostasis. ***Infect Immun*** 1999; 67 (7): 3610- 8.
2. Maes R. Clinical usefulness of serological measurements obtained by antigen 60 in mycobacterial infections: development of a new concept. ***Klin Wochenschr*** 1991; 69 (15): 696- 709.
3. Glatman-Freedman A, Casadevall A. Serum therapy for tuberculosis revisited: reappraisal of the role of antibody-mediated immunity against Mycobacterium tuberculosis. ***Clin Microbiol Rev*** 1998; 11(3):514-32.
4. Mehrotra J, Bisht D, Tiwari VD, Sinha S. Serological distinction of integral plasma membrane proteins as a class of mycobacterial antigens and their relevance for human T cell activation. ***Clin Exp Immunol*** 1995; 102 (3): 626-34.
5. Fifis T, Costopoulos C, Radford AJ, Bacic A, Wood PR. Purification and characterization of major antigens from a Mycobacterium bovis culture filtrate. ***Infect Immun*** 1991; 59 (3): 800-7.
6. Pal PG, Horwitz MA. Immunization with extracellular proteins of Mycobacterium tuberculosis induces cell-mediated immune responses and substantial protective immunity in a guinea pig model of pulmonary tuberculosis. ***Infect Immun*** 1992; 60 (11): 4781-92.
7. Tantimavanich S, Nagai S, Nomaguchi H, Kinomoto M, Ohara N, Yamada T. Immunological properties of ribosomal proteins from Mycobacterium bovis BCG. ***Infect Immun*** 1993; 61 (9): 4005-7.
8. Hubbard RD, Flory CM, Collins FM, Cocito C. Immunization of mice with the antigen A60 of Mycobacterium bovis BCG. ***Clin Exp Immunol*** 1992; 88 (1): 129-31.
9. Gilot P, Coene M. Thermostable macromolecular antigens from mycobacteria. ***Can J Microbiol*** 1994; 40 (8): 605-11.
10. Cavalcante S, Kritski AL, Ferreira MA, Souza MA, Laszlo A, Werneck-Barroso EB, et al. Association between an early humoral response to Mycobacterium tuberculosis antigens and later development of tuberculosis in human immunodeficiency virus-infected individuals. ***Int J Tuberc Lung Dis*** 1997; 1 (2): 170-4.

11. Costello AM, Kumar A, Narayan V, Akbar MS, Ahmed S, Abou-Zeid C, et al. Does antibody to mycobacterial antigens, including lipoarabinomannan, limit dissemination in childhood tuberculosis? ***Trans R Soc Trop Med Hyg*** 1992; 86 (6): 686- 92.
12. NEWBOULD BB. CHEMOTHERAPY OF ARTHRITIS INDUCED IN RATS BY MYCOBACTERIAL ADJUVANT. ***Br J Pharmacol*** 1963; 21:127-36.
13. Rennou M, Maes M.C, Maes H.H, Maes R.F, Kidwai Z, Tasbiti H, et al. Mycobacteria antibodies I. Antibodies against BCG and M. tuberculosis H37 RA do not consistently recognize pathogenic M. tuberculosis cells but recognize their cytoplasmic constituents. This journal.
14. Cocito C, Vanlinden F. Preparation and properties of antigen 60 from Mycobacterium bovis BCG. ***Clin Exp Immunol*** 1986; 66 (2): 262-72.
15. Cocito C, Vanlinden F. Subcellular localisation and sedimentation behaviour of antigen 60 from Mycobacterium bovis BCG. ***Med Microbiol Immunol (Berl)*** 1988; 177 (1): 15-25.
16. Hamasur B, Kallenius G, Svenson SB. A new rapid and simple method for large-scale purification of mycobacterial lipoarabinomannan. ***FEMS Immunol Med Microbiol*** 1999; 24 (1): 11-7.
17. Maes RF, Claverie N. The effect of preparations of human chorionic gonadotropin on lymphocyte stimulation and immune response. ***Immunology*** 1977; 33 (3): 351-60.
18. McIntyre G, Stanford JL. Immunodiffusion analysis shows that Mycobacterium paratuberculosis and other mycobactin-dependent mycobacteria are variants of Mycobacterium avium. ***J Appl Bacteriol*** 1986; 61 (4): 295-8.
19. Bennett JA, Gruft H, McKneally MF, Zelterman D, Crispen RG. Differences in biological activity among batches of lyophilized tice Bacillus Calmette-Guerin and their association with clinical course in Stage I lung cancer. ***Cancer Res*** 1983; 43 (9): 4183-90.
20. Beschin A, Brijs L, De Baetselier P, Cocito C. Mycobacterial proliferation in macrophages is prevented by incubation with lymphocytes activated in vitro with a mycobacterial antigen complex. ***Eur J Immunol*** 1991; 21 (3): 793-7.
21. Mustafa AS. Mycobacterium bovis BCG-induced Th1 type CD4+ suppressor T cells act by suppressing IL-2 production and IL-2 receptor expression. ***Nutrition*** 1995; 11 (5 Suppl): 692-4.
22. Schauf V, Rom WN, Smith KA, Sampaio EP, Meyn PA, Tramontana JM, et al. Cytokine gene activation and modified responsiveness to interleukin-2 in the blood of tuberculosis patients. ***J Infect Dis*** 1993; 168 (4): 1056-9.
23. Chan J, Fan XD, Hunter SW, Brennan PJ, Bloom BR. Lipoarabinomannan, a possible virulence factor involved in persistence of Mycobacterium tuberculosis within macrophages. ***Infect Immun*** 1991; 59 (5): 1755-61.
24. Teitelbaum R, Glatman-Freedman A, Chen B, Robbins JB, Unanue E, Casadevall A, et al. A mAb recognizing a surface antigen of Mycobacterium tuberculosis enhances host survival. ***Proc Natl Acad Sci U S A*** 1998; 95 (26): 15688-93.
25. Glatman-Freedman A, Mednick AJ, Lendvai N, Casadevall A. Clearance and organ distribution of Mycobacterium tuberculosis lipoarabinomannan (LAM) in the presence and absence of LAM-binding immunoglobulin M. ***Infect Immun*** 2000; 68 (1): 335-41.
26. Maes HH, Causse JE, Maes RF. Tuberculosis I: a conceptual frame for the immunopathology of the disease. ***Med Hypotheses*** 1999; 52 (6): 583-93.
27. Von Reyn CF, Marsh BJ, Waddell R, Lein AD, Tvaroha S, Morin P, et al. Cellular immune responses to mycobacteria in healthy and human immunodeficiency virus-positive subjects in the United States after a five-dose schedule of Mycobacterium vaccae vaccine. ***Clin Infect Dis*** 1998; 27 (6): 1517-20.