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Mycobacterial Antibodies Against BCG and M.Tuberculosis H37RA Do Not Recognize Pathogenic M.Tuberculosis Cells but Do Recognize Their Cytoplasmic Constituents

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ABSTRACT

The humoral immune response against mycobacterial pathogens and BCG vaccine is still poorly understood. It is not known if antibodies raised against BCG react with all pathogenic M. tuberculosis strains. The question arose during the development of a tuberculosis detection system based on antibodies. It was found that antibodies raised against cells of avirulent TB strain H37Ra and against whole sonicates of BCG do not react consistently with whole cells of pathogenic TB strains and very poorly with whole BCG cells. These antibodies; however, react very well with the internal components of the BCG cell (i.e. A 60 and cytoplasmic constituents) ***(Tanaffos 2006 5(4): 15-21)****.*

Key words: *Vaccination, Mycobacterium tuberculosis, BCG, Immunosuppression antibodies*

INTRODUCTION

We are presently developing a flow-through method for the detection of the tuberculosis pathogen to provide a sensitive detection method at an affordable price in regions where the prevalence of tuberculosis is high. It is based on labelling the bacilli with antibodies raised against the cells of avirulent TB strain H37Ra. The new method tested in the field gave equivocal results with sputum samples shown positive by AFB staining.

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Investigation of this unexpected lack of sensitivity was carried out on all components of the kit, as on outside influences such as travel and storage conditions. Our data, based on an analysis of humoral antibodies elicited by various mycobacterial antigens, showed that the phenotypic variation of the outer membrane of the TB pathogen is the likely cause of the variable efficacy of the developed test. This variation could be an important element to take into consideration when devising a vaccine against TB.

MATERIALS AND METHODS

The flow-through method was used and the bacilli present in a liquid sample were filtered through a

0.45µm nitrocellulose membrane (Advanced Microdevices, Ambala, Cannt, India). The bacteria retained on the surface of the membrane are labelled with rabbit antibodies against whole mycobacterial cells. The presence of rabbit gammaglobulins is visualized on the membrane with gold-labelled protein A.

Bacilli filtered on the retaining membrane were:

1. Freeze-dried inactivated bacilli of *M. tuberculosis* strain H37Ra (from Difco, France).
2. Pathogenic bacilli from fresh sputum of TB positive patients from Tehran.
3. Pathogenic bacilli isolated by centrifugation from the sputum of TB patients diagnosed in Paris.
4. Live biomass of BCG bacilli, obtained from the "Institut Pasteur du Brabant" (PIB).
5. Freeze-dried live BCG bacilli (from Aventis-Pasteur Monovax lot W5586-2).
6. Live cultured pathogenic bacilli (from the sputum of patients diagnosed in Tehran).

Other patient materials:

- Fresh sputa from non-TB patients (courtesy of Dr. Fraisse, CHU Hautepierre, France). One of these samples was determined by our method to be positive, after reception.
- Frozen sputa from non-TB patients (courtesy of Dr. D. Banerjee, St. George's Hospital Medical School, London). Frozen sputa from Brazilian non-TB and TB patients (courtesy of Dr.M. Perkins from WHO, Geneva, Switzerland).
- Fresh sputa, positive and negative (analyzed on site at the Pasteur Institute of Iran, the laboratory of Dr. Ehsanullah, Karachi, Dr. S. Gokhale and M. Singh at the Command Hospital, Lucknow).
- Samples from diverse biological fluids (analyzed on site by Dr. Gokhale at the Command Hospital, Lucknow).
- CRF fluids (analyzed on site by Pr. Chandramucki at the NIMHANS in Bangalore, India).

Non-TB patients sputa were used for spiking purposes and determination of background adsorption. Homogenized cell suspensions of cultures and other biomasses were obtained by suspending the material in isotonic saline and shakingly vigorous. The concentration of bacilli was evaluated by the classical AFB stain.

The number of bacterial cells used for determination of the specificity of antibodies for strain H37Ra, in the test was adjusted to 1 bacillus per microscopic field, corresponding to $2.3x10^6$ bacteria/ ml.

Fifty microliters of this aqueous suspension (i.e. $1.15x10^5$ bacteria) was passed through the retaining membrane. For evaluation of the specificity of the antibodies against the two BCG strains, the concentration of the bacilli was increased to 50 bacilli per microscopic field, corresponding to $1.15x\ 10^8$ bacilli/ ml. Fifty microliters of the bacterial suspension, i.e. $5.7x10^6$ bacteria, were passed through the retaining membrane. For cultured pathogenic strains of TB, the concentration was adjusted to 1 McFarland turbidity ($3x\ 10^8$ cells/ ml) and 50µl, corresponding to $1.5x\ 10^7$ bacilli, which were used in the test. All of the above were used either as is, or spiked in TB-free sputa. For the pathogenic bacilli in sputa, samples were used that contained more than 10 bacteria per microscopic field, as determined by AFB staining. Fifty microliters of sputum, i.e. more than $1.15x\ 10^6$ bacteria were dissolved in N-acetyl-cysteine/ NaOH and passed through the retaining membrane. Tuberculosis strain H37Ra at a concentration of 1-2 bacilli per microscopic field was spiked in TB-negative sputa and processed in the same manner and used as controls.

Sensitization of the membrane with mycobacterial antigens was done by spotting 5 micrograms of protein on the membrane and drying. The antigens

used to sensitize the membrane were:

1. Antigen 60 (A60) from M. bovis, strain BCG obtained from PIB.
2. Cytoplasm from M. bovis, strain BCG obtained from PIB.
3. Cytoplasm from heat-inactivated wild TB strain.

These antigens were obtained by cell disruption and, in the case of the A60 complex, chromatographic isolation by standard methods (1). Heat- inactivation of bacteria was achieved by incubating the suspension of cells for 60 minutes at 80ºC. No positive cultures were obtained from the inactivated bacilli. The inactivated bacteria appeared whole under the microscope.

Antibodies used to label the cells and the antigens retained on the membrane were:

1. Rabbit antibodies against inactivated freeze-dried *M. tuberculosis* strain H37Ra (Difco 231141), (Neosystem, France).
2. Rabbit antibodies against a whole sonicate of the Copenhagen strain of BCG (Dako, Denmark).
3. Rabbit antibodies against A60 of BCG (Neosystem, France).
4. Rabbit antibodies against whole heat-inactivated pathogenic bacilli obtained from the Pasteur Institute of Iran (Neosystem, France).

Immunizing solutions for the production of antibodies by Neosystem were provided by the authors and consisted of $3.75x10^8$ heat- inactivated pathogenic TB cells in 500 µl of physiological solution and 450µg of *M. tuberculosis* H37Ra inactivated freeze-dried cells in 500 µl of physiological solution. Both vaccines were homogenized with an equal amount of Freund's incomplete adjuvant and inoculated monthly.

The antisera were precipitated with ammonium sulfate, dissolved in a tenth volume phosphate-buffered saline, dialyzed and clarified by low speed centrifugation. The working dilution of the primary rabbit antibodies was evaluated by the reactivity of different concentrations of these antibodies with whole cells of *M. tuberculosis* H37RA spotted on the membrane. The rabbit antibodies suitably diluted in an isotonic medium containing 1% bovine albumin and 0.1% tween 20 at pH 7.2 were contacted (80 µl) with the sensitized membrane and the antibodies attached to the sensitizing antigens were revealed with 80µl of a suitably diluted protein A- Gold (40 nanometers) solution purchased from British Biocell (UK). The intensity of the red coloration, indicative of the presence of rabbit gammaglobulins on the membrane, was visually estimated with a gradation extending from "-" absence of coloration to "++++", indicative of intense coloration. The limit of sensitivity was signaled by "+/-".

We evaluated the interaction of antibodies with bacilli of tuberculosis strain H37Ra, cultured pathogenic TB bacilli originating from the Pasteur Institute of Iran, live BCG from Aventis, and cytoplasm as well as A60 of BCG. The results obtained with the other pathogenic bacilli were restricted to their interaction with antibodies against strain H37Ra and were used essentially to verify the phenotypic variation of pathogenic strains. These antigens were not available for additional studies with other antibodies.

RESULTS

The antibodies produced in rabbits against bacilli of the *M.tuberculosis* H37Ra strain were abundant and allowed the set-up of the flow- through test wherewith three antibodies and various antigens were tested. A number of sputa collected from TB patients living in different parts of the world were analyzed. The size of the samples was not in our power to define. The results of this analysis are given in table 1.

We observed no difference in response whether the sputa were used fresh or thawed. The failure of antibodies raised against bacilli of strain H37Ra to

recognize pathogenic strains in biologic samples was confirmed in Bangalore by Pr. Chandramucki who found no response when analyzing CSF samples from tuberculous meningitis patients. The absence of recognition of pathogenic strains and BCG by anti-whole cell H37Ra and their poor recognition by anti-sonicate BCG antibodies was confirmed by the analysis of 9 culture-isolated strains obtained from the Pasteur Institute of Iran, grown on Lowenstein medium and suspended in buffered physiological saline at one McFarland turbidity. No immunological reaction was observed with antibodies against H37Ra, and only 3 samples were poorly recognized (score -/+) with anti-BCG. The two BCG's were concentrated in saline to 50 bacteria per microscopic field for the evaluation of their immunological reactivity.

Table 1. Results of flow-through tests on positive samples from various origins using 3 different detection antibodies.

	Samples		Antibodies against		
			H37Ra	A60	BCG
TB	H37Ra	1.15x 10^5 bact.	++++	+++	++++
	France	1 patient	+++	...*	...
	Br	12 patients	++ to ++++	...	...
	India extrapul	30 Patients	+ to ++++	...	...
	India	50 patients	- to +++	...	...
	Iran	50 patients	-	...	...
	Iran **	1.5x10^7 bact. 9 patients	-	...	...
	France ***	12 patients	-	...	...
	Pakistan	5 patients.	-	...	...
BCG	PIB	5.7x10^6 bact.	+/-	+/-	+/-
	Aventis	5.7x 10^6 bact.	+/-	+/-	+/-
	A60	5 micrograms	++++	++++	++++
	Cytopl.	5 micrograms	++++	+++	++++

*: Not done

**: Cultured samples, analyzed at a concentration of 1 McFarland in buffer.

***: Concentrated sediment suspended in buffer after decontamination.

These results were at odds with previous observations of positivity of a smear-positive sputum sample obtained in Strasbourg, of 12 smear-positive sputa from Brazilian TB patients, and also in diverse samples analyzed in Lucknow. Interestingly, a fairly good response was obtained there in extra-pulmonary TB specimens but equivocal results with sputum, in that the positivity of sputa asserted by microscopic examination was sometimes, but not always confirmed by the flow- through method.

We verified the immunological reactivity of antibodies produced in rabbits against a heat-inactivated strain of pathogenic bacilli. The antibody recognized A60 (score: ++++) as well as whole cells of TB strain H37Ra (score: ++++), but less efficiently the cells of its own immunizing strain (score: ++), and scarcely BCG (Aventis) (+/-). It recognized very efficiently (score: ++++) the cytoplasm of the lysed cells of the wild strain used for immunization. After centrifugation for 30 minutes at 16,000 rpm, the immunological activity of the lysed cells remained confined to the supernatant (++++) with little immunological reactivity observable in the resuspended sediment, containing cell debris and cell membranes.

The immunological specificity of the cell membranes of the different mycobacterial entities here analyzed could also be a property of A60 and the cytoplasm. This was suggested by the slightly reduced reactivity of antibodies against A60 extracted from BCG H37Ra cells (table 1). The large amount of A60 used as a capture antigen (5µg) had been applied to unequivocally establish the interaction of the antibodies with it but could have been too great to assess any difference in specificity of the different antibodies analyzed. To investigate this possibility, the cytoplasm of a wild Iranian strain of TB, used to raise antibodies, and the cytoplasm of BCG (PIB) were spotted on a membrane and the reactivity of antibodies against the wild strain, against H37Ra, and against A60 from BCG (PIB) were evaluated in a flow-through system. The results are given in table 2.

Table 2. Reactivity of 3 different antibodies with the cytoplasm of a wild pathogenic TB strain and the cytoplasm of BCG.

Antiserum against	Cytoplasm wild strain	Cytoplasm BCG
Wild strain	++++	+/-
H37Ra	+++	+/-
A60 (BCG)	+ +/-	+++/-

The intensity of the coloration obtained with antiserum against A60 of BCG was significantly lower (score between + and ++) and significantly higher (score between ++ and +++) than those obtained with the antibodies raised against TB strains, yet were not due to a difference in concentration of antibodies applied. The observed differences indicated a difference in specificity.

DISCUSSION

The lack of sensitivity of the test under development was not due to an inactivation of one of its components, since we obtained good sensitivity with positive samples in some cases. This was confirmed by a battery of tests, not presented here, on the different components of the test. The problem was traced to a lack of specificity of the primary rabbit antibodies used. They were raised against avirulent TB strain H37Ra and did not consistently recognize pathogenic strains. These results indicate an unexpected antigenic variation of the cell surface of wild tuberculosis strains. Also new to us, was the failure of BCG cytoplasm antibodies to recognize whole BCG cells as efficiently as their cytoplasmic constituents.

Table 1 reveals (first column) that the anti- *M. tuberculosis* H37Ra antibodies recognize bacilli of strain *M. tuberculosis* H37Ra very well, but very poorly recognize BCG bacilli, and do not recognize pathogenic *M. tuberculosis* strains originating from some cities, with equivocal results in Lucknow and good results elsewhere. Recognition of the inner components (cytoplasm and A60) of the BCG cell is noted.

In mice, BCG induced a higher level of anti- BCG antibodies when the bacilli are grown in asparagine-containing medium, versus starch- and peptone containing medium (2). This effect of growth medium on immunogenicity cannot explain our observations, since the failure of recognition was observed when TB-positive sputa were directly analyzed, when bacilli were isolated by centrifugation from sputum and resuspended in saline and when bacilli cultured on Lowenstein medium were washed and suspended in isotonic saline. But was not observed when TB strain H37Ra was analyzed, either suspended in sputum or in saline. The condition of the cells, namely fresh, frozen, or freeze-dried was not at cause either, because freeze-dried H37Ra cells were recognized by all 4 antibodies, freeze-dried BCG was not and neither were many positive fresh and frozen sputum samples.

As with antibodies directed against whole H37Ra cells, antibodies against A60 of BCG (second column of table 1) and against whole sonicates of BCG (third column) poorly recognized whole BCG cells (PIB and Aventis: score+/-). By contrast, they recognized the cytoplasmic constituents of BCG (score +++ and ++++) and also H37Ra cells, which is puzzling. Antigen 60 was itself, recognized by all four antibodies used in this study. It; thus, seems that this antigen 60 would be accessible late during an infection (3), when host cells lyse. This observation confirms that A60 is mainly located intracellularly (4). Yet, anti-A60 antibodies recognize whole cells of avirulent tuberculosis strain H37Ra indicating that this strain H37Ra presents on its surface much more of the constituents of A60, which is a complex bearing at least 30 antigenic constituents and presenting about 85 particular epitopes. This is contrary to all other strains tested here, where the presence of A60 constituents on the surface would be

too low to be evidenced by our method of detection.

Noteworthy is that the stained cells of strain H37Ra appeared as slightly bent rods under the microscope which set it completely apart from all other strains analyzed here, be they pathogenic or attenuated, that appeared coccoidal.

It is tempting to recognize a geographical pattern of sensitivity. Samples from Brazil are detected by anti- H37Ra antibodies while those from Karachi and Tehran are not detected by any of the applied anti bodies. Both types seem to occur in India and France. The evidence is still scant. We assume that the reasons for this variation are multiple and hard to pinpoint at this stage. One may advance the premature abandon of treatment by patients, which favors the growth of resistant strains that may present alterations of their cell surface. These alterations may vary according to the regimen applied (5). Another source of variation that could be operative among the Hindu population of India is the consumption of raw milk from infected cows.

Our concern in this investigation was to understand the failure of the test in this field. The choice of antibodies against H37Ra (or BCG as a substitute) for the development of a diagnostic test was dictated by our desire to base this test on harmless biologics. The literature on mycobacteria gives little indication that antibodies play a significant role during infection and we did not expect the results we obtained.

Some of the data obtained during this investigation indicate that humoral antibody production may play a crucial role in the outcome of a mycobacterial infection or vaccination. H37Ra TB strain was very effective in the creation of antibodies against its own cell surface (Table 1). This reactivity may be the reason for the avirulence of H37Ra. This reactivity was not found with antibodies against the BCG, which recognized H37Ra cells but neither PIB nor Aventis cells. Antibodies against a virulent TB strain did not readily react with BCG cytoplasm (Table 2). In addition, the A60 of BCG elicited antibodies that are not totally specific for the homologous component of pathogenic bacilli. This data supports the idea that BCG vaccination does not effectively protect against tuberculosis because it induces the production of antibodies mainly against antigens only exposed when cells are lysed and when the infection is well established. This is a simple explanation why BCG does not protect against infection but prevents dissemination from the primary foci to other parts of the body (6). The data further indicates that geographical variations in exposed antigens among *M. tuberculosis* strains may be the cause of the discrepant results found when the efficacy of various BCG strains is analyzed in different parts of the world. These subjects will be studied in the following communications.

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