INFECTION AND IMMUNITY, May 1973, p.777-780

Vol. 7, No. 5
Printed in U.S.A.

Comparison of Strains of BCG

I. Antigenic Analysis and Tuberculin Reactivity

SOTIROS D. CHAPARAS AND SALLY R. HEDRICK

Bureau of Biologics, Food and Drug Administration, Rockville, Maryland 20852

Received for publication 30 January 1973

At least 10 of 11 demonstrable antigens in unheated culture filtrates of 12 vaccine strains of BCG were shared as determined in a reference antigen-antibody immunoelectrophoresis system. Filtrates from each of the BCG strains gave equivalent skin test reactions in homologously and heterologously sensitized guinea pigs. By these immunological parameters, the 12 BCG strains were remarkably similar.

BCG vaccines have been used for many years to induce immunity to tuberculosis. The potential of various mycobacterial species as adjuvants to heighten immune responses to other antigens has also been recognized. Mackaness (6) pointed out an element of nonspecific immunity due to BCG vaccines whereby protection toward unrelated organisms was noted. This nonspecific immunity was of shorter duration than a concommittant specific immunity. More recently, BCG vaccines were shown to be useful in immunotherapy of experimental and clinical cancer systems (8, 9). Strains of BCG currently used for vaccination against tuberculosis throughout the world and for experimental cancer immunotherapy stem from the original Bacille Calmette-Guérin, L'Institut Pasteur, Paris. However, among these strains which were handled under different conditions, differences were reported in the antigenic content (5), sensitizing potency (12), cord formation (10), biochemical reactivity (1), the ability to protect against challenge with tubercle bacilli, and virulence (4). In the present study a comparison was made of the antigenic content of culture filtrates of 12 BCG strains and the ability of each of these strains to induce sensitivity to homologous and heterologous tuberculins.

MATERIALS AND METHODS

A reference antiserum and antigen preparation for the H37Rv strain of *Mycobacterium tuberculosis* was described (3) and obtained from the U.S.-Japan Tuberculosis Program, National Institute of Allergy and Infectious Diseases, Bethesda, Md. The 12 strains of BCG were obtained from the Trudeau Institute in Saranac Lake, N.Y., and are described in Department of Health, Education, and Welfare publication no. (NIH) N-289. Most of the cultures were obtained from various countries and were transferred about three times to build up seed material.

The harvested organisms were suspended in Middlebrook 7H-9 Tween-albumin broth and stored at below −70 C. Tuberculin-like preparations were made by growing the organisms on synthetic medium of Long. Depending on the appearance of the pellicle, as an indication of autolysis, unheated culture filtrates were harvested 3 to 5 months after inoculation. The culture filtrates were completely dialyzed against distilled water and then freeze-dried. A few cultures of each strain were harvested after 1 month of growth. These organisms were killed by heating at 100 C for 10 min, then dried, and used in sensitizing preparations. Electrophoresis was performed in 1% agar on glass slides (1 by 3 inch; 2.54 by 7.62 cm) with Veronal electrophoretic buffer No. 2 (Fisher Scientific Co.), pH 8.6, ionic strength 0.075. Separation of antigens was performed at 20 V and 6.5 mA per slide for 80 min. Reference antiserum was added to cut channels and permitted to diffuse toward the separated antigens. Photographs were taken at 48 h. For identification of bands, a cutter was used with two antigen holes between the antibody channels. This permitted the reference antigen and the test antigen to migrate side by side, and lines of identity were produced after diffusion of antiserum.

For skin testing, 12 Hartley strain female guinea pigs between 300 and 400 g were used for each species. Each animal received subcutaneously in the nuchal area 0.2 ml of an emulsion containing 10 mg of organisms. The emulsions were made of equal parts of oil phase (Arlacel A-Drakeol 6 VR, 35:65) and saline as described previously (2). Six weeks later, a preliminary titration was performed with four of the sensitized guinea pigs in each group to select a dose which would produce at 24 h an area of erythema with a diameter of approximately 14 mm. Each of the remaining eight guinea pigs from each group received 0.1 ml of the selected doses of all 12 antigens. Reactions were read at 24 h. Skin test data were analyzed by the method of Magnusson (7) which is based on specificity of delayed skin reactions for

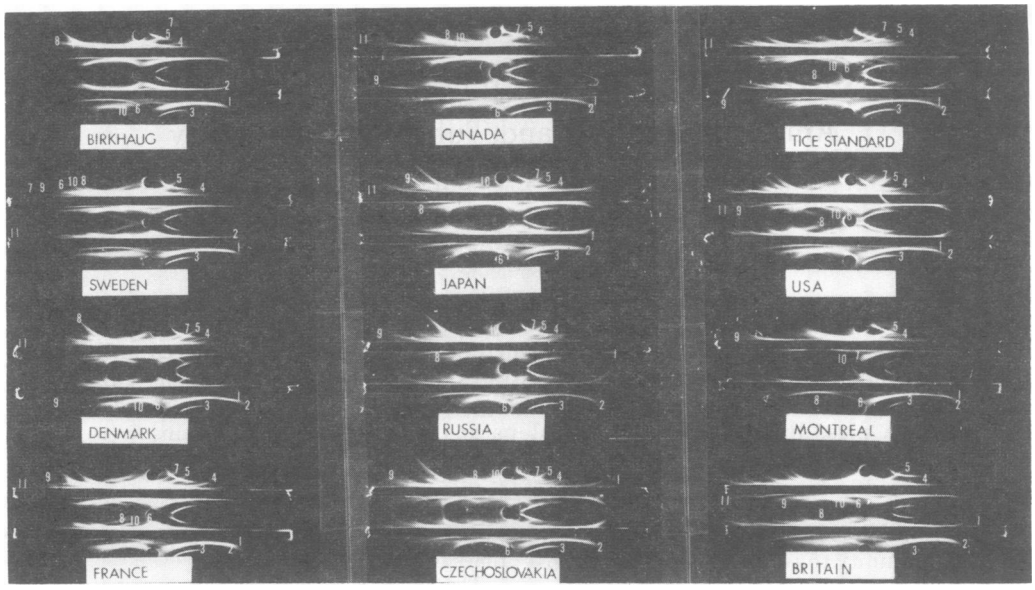

FIG. 1. *Immunoelectrophoretic patterns of culture filtrates from 12 strains of BCG developed with reference antiserum. Top, middle, and bottom wells were filled with solutions containing 50, 25, and 10 mg, respectively, of total solids per 1.0 ml. Not all bands were visible photographically.*

differentiating among various species of mycobacteria.

RESULTS

Immunoelectrophoretic patterns for the antigens detected in the 12 BCG culture filtrates are shown in Fig. 1, and the presence or absence of antigens is tabulated in Table 1. Proper identification for a number of precipitin bands with those of the standard reference system was confirmed with a special cutter as described above and as illustrated in Fig. 2 for the antigens of the Japanese strain of BCG. Fusion of some bands was very faint and could not be photographed. It was not possible to get bands of identity for some of the bands. However, the rate of electrophoretic migration and the degree of arcing strongly suggested identity of bands to the reference. Complete resolution of bands could best be made by using three antigen concentrations: 50, 25, and 10 mg per ml. The polysaccharides (bands 1, 2, and 3) were best discriminated at the lowest concentration, whereas the protein antigens were best discriminated at the highest concentration. The precipitin bands were numbered by a reference system described previously (3) and referred to above. Bands 1, 2, and 3 migrated cathodally and represented polysaccharide antigens. Bands 1 and 2 were identified as antigens contained in Seibert's polysaccharide I, and band 3 represented Seibert's polysaccharide II

TABLE 1. *Identification of antigens in BCG culture filtrates*

BCG strain	Reference band no.										
	1	2	3	4	5	6	7	8	9	10	11
USA	+[a]	+	+	+	+	+	+	+	+	+	+
Montreal	+	+	+	+	+	+	+	+	+	+	
Japan	+	+	+	+	+	+	+	+	+	+	+
Tice	+	+	+	+	+	+	+	+	+	+	+
France	+	+	+	+	+	+	+	+	+	+	+
Russia	+	+	+	+	+	+	+	+	+	+	
Czech	+	+	+	+	+	+	+	+	+	+	
Birkhaug	+	+	+	+	+	+	+	+		+	+
Canada	+	+	+	+	+	+	+	+	+	+	+
Sweden	+	+	+	+	+	+	+	+	+	+	+
Denmark	+	+	+	+	+	+	+	+	+	+	+
Britain	+	+	+	+	+	+	+	+	+	+	+

[a] Indicates the presence of a precipitin band. Intensity of bands varied but is not reflected in this table.

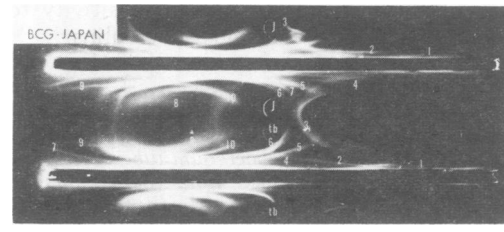

FIG. 2. *Identification of precipitin bands produced with culture filtrate of Japan BCG (25 mg/1.0 ml, upper two wells) with those produced with the H37Rv reference system (lower two wells).*

(11). All culture filtrates contained bands 1 to 8 and band 10. Only the Birkhaug strain lacked band 9. Band 11 could not be demonstrated in three filtrates (Russian, Czechoslovakian, and Montreal). Skin test data were analyzed by the method of Magnusson (7) which was based on specificity of delayed skin reactions for differentiating between mycobacteria. Specificity differences (SPD) between homologous and heterologous reactions were used to relate and differentiate species: SPD = (Aa + Bb) − (Ab + Ba) (see Table 2). By this method of analysis for any two species, the SPD should be smaller the more closely related the species are. SPD values of 3 or less indicate similarity. Thus, the observed SPD values in Table 2 for challenges between any two species did not exceed 1.6, implying a very close relationship.

DISCUSSION

One of the most striking observations of this study is the great antigenic similarity of the various BCG tuberculins. The 12 BCG filtrates were compared and found to share almost all of the antigens of the reference system. Inasmuch as the reference system was for *M. tuberculosis*, the supposed antigenic similarity of BCG strains to the human tubercle bacillus was reinforced. BCG strains from all over the world, which were grown in different laboratories under different conditions, maintained virtually the same antigens demonstrable by the reference. In fact, antisera prepared against any of the BCG filtrates and organisms contained fewer precipitins and none different from the reference. However, differences were shown among these strains from the standpoint of cord formation (10), virulence for hamsters (4), immunizing capacity (4), and biochemical characterization (1). Thus, antigenic comparisons with the reference system reflect stable characteristics of the organisms. In this study an effort was not made to quantitate the amount of each antigen present in culture filtrates of the mycobacteria, but it is obvious from Fig. 1 that quantitative differences do exist. In general, the carbohydrates could be satisfactorily identified at lower concentrations than the proteins. Small concentrations of certain substances could only be demonstrated in concentrated filtrates. Culture filtrates of concentrations greater than 50 mg/ml were not used because if this amount of material were necessary to demonstrate an antigen, it was probably due to a minor, but not necessarily unimportant, component. Further homogeneity could be seen among the various strains by analyses of skin tests in guinea pigs sensitized with the homologous or heterologous strains of BCG. Magnusson (7) successfully employed the skin sensitivity reaction for comparison of strains, with the use of a formula to determine specificity differences. The remarkably small specificity differences found in the present studies indicate that each antigen preparation reacted as well in guinea pigs sensitized with any of the strains of BCG as in the homologously sensitized group.

Thus, by at least two immunological parameters, marked similarities were observed among 12 strains of BCG. Since differences among BCG strains in their abilities to convey protection against virulent challenge (4) were reported, it appears that protective efficacy, skin sensitivity, and other immune responses induced by strains may not necessarily be correlated. Nevertheless, the antigenic similarities in strains of BCG maintained over many generations under various conditions are remarkable.

TABLE 2. *Specificity differences (SPD)[a] of antigens from various strains of BCG*

	USA	Mon	Jap	Tice	Fra	Rus	Cze	Bir	Can	Swe	Den	Glax
USA[b]	—											
Mon	0.6	—										
Jap	1.3	1.3	—									
Tice	0.5	0.9	1.3	—								
Fra	0.4	0.5	1.5	0.6	—							
Rus	0.2	0.2	0.3	0.3	1.0	—						
Cze	0.4	−0.2	1.4	1.1	1.4	1.1	—					
Bir	0.2	1.0	0.2	0.7	0.5	0	1.3	—				
Can	0.6	0.5	1.4	1.1	1.6	0.1	1.0	0.2	—			
Swe	0.4	−0.2	1.2	0.4	0	0.2	1.1	0.4	0.9	—		
Den	1.0	0.5	1.5	0.1	0	1.5	0	0.7	0.8	0.6	—	
Glax	−0.4	0	−0.1	0.2	−0.3	−0.3	0.7	0.3	−0.5	−0.9	0.1	—

[a] SPD = (Aa + Bb) − (Ab + Ba). Abbreviations: Aa, diameter of homologous A reaction; Bb, diameter of homologous B reaction; Ab, diameter of heterologous A reaction; Ba, diameter of heterologous B reaction.

[b] See Table 1 for complete names of strains listed.

LITERATURE CITED

1. Bönicke, R. 1957. The classification of mycobacteria with the help of different chemical tests. Bull. Int Un. Tuberc. **27**:151-166.
2. Chaparas, S. D., and H. Baer. 1964. The immunology and chemistry of tuberculin. II. Chromatography with Sephadex of the nondialyzable tuberculin active constituents of BCG culture filtrate. Amer. Rev. Resp. Dis. **89**:41-48.
3. Janicki, B. W., S. D. Chaparas, T. M. Daniel, G. P. Kubica, G. L. Wright, and G. S. Yee. 1971. A reference system for antigens of *Mycobacterium tuberculosis*. Amer. Rev. Resp. Dis. **104**:602-604.
4. Jespersen, A., and M. W. Benton. 1963. The acquired resistance to tuberculosis induced by BCG vaccine assayed by a quantitative method on red mice. II. Vaccination effect of BCG strains strongly or moderately virulent for hamsters. Acta Tuberc. Pneunol. Scand. **44**:276-289.
5. Lind, A. 1960. Serological studies of mycobacteria by means of diffusion-in-gel techniques. III. A difference in precipitinogenic content found in substrains of BCG. Arch. Allergy **17**:1-9.
6. Mackaness, G. B. 1971. Resistance to intracellular infection. J. Infect. Dis. **123**:439-445.
7. Magnusson, M. 1971. A comparative study of *Mycobacterium gastri* and *Mycobacterium kansasii* by delayed type skin reactions in guinea pigs. Amer. Rev. Resp. Dis. **104**:377-384.
8. Mathé, G. 1971. Experimental doses and first clinical controlled trials of leukemia active immunotherapy. Progress in Immunology—First Intern. Cong. Immunol., p. 959-969. Academic Press Inc., New York.
9. Pearson, J. W., G. R. Pearson, W. T. Gibson, J. C. Chermann, and M. A. Chirigos. 1972. Combined chemoimmunostimulation therapy against murine leukemia. Cancer Res. **32**:904-907.
10. Rosenthal, S. R. 1957. BCG vaccination against tuberculosis, p. 69-83. Little Brown & Co., Boston.
11. Seibert, F. B. 1949. The isolation of three different proteins and two polysaccharides from tuberculin by alcohol fractionation. Their chemical and biological properties. Amer. Rev. Tuberc. **59**:86-101.
12. World Health Organization expert committee on biological standardization. 1966. Requirements for dried BCG vaccine. W.H.O. Tech. Rep. Ser. **329**:25-49.