Volume 288, number 1,2, 77–80 FEBS 10049 August 1991

ADONIS 001457939100757B

A 64 kDa protein from *Mycobacterium bovis* BCG shares the same antigenic determinants with line 10 hepatoma cells and has anti-line 10 tumor activity

C.R. Ahsan and J. Sasaki

Department of Bacteriology, Hirosaki University School of Medicine, Hirosaki 036, Japan

Received 24 May 1991

A 64 kilodalton (kDa) surface protein was isolated from the water-extracted materials from *Mycobacterium bovis* strain BCG, the determinants of which are antigenically shared by a 64 kDa major surface antigenic component of line 10 hepatoma cells. The 64 kDa protein showed anti-line 10 tumor activity in pre-immunized guinea pigs, and this suggests that the BCG 64 kDa protein is probably identical with the tumor specific antigen.

Tumor immunotherapy; Surface antigen; Hepatoma cell; *Mycobacterium bovis*

1. INTRODUCTION

A number of studies have attempted to purify and characterize species-specific immunologically active mycobacterial antigens associated with the host cellular immune responses [1–4]. However, one particular antigen from *Mycobacterium bovis* BCG, designated the 64 kilodalton (kDa) antigen, has received much attention because it appears to be one of the major soluble, immunologically active protein antigens [5–8]. Purified 64 kDa antigen also elicited a strong delayed type hypersensitivity reaction in experimental animals and promoted a significant level of IgG antibody in sensitized guinea pigs [7].

M. bovis BCG has also been investigated widely within the scope of cancer immunotherapy of experimental animals and humans [9–12]. Much effort has also been made to isolate the specific components possessing antitumor activity from BCG [13–15], and there are reports on the antigenic relationships between BCG and different animal tumor and human cancer cells [16–18].

Recently we described the presence of common antigenic determinants between surface extracts of line 10 tumor cells (and others) and BCG, using BCG monoclonal antibodies [19]. In this paper, we demonstrate a 64 kDa protein from BCG, which shares common antigenic determinants with line 10 tumor cells and has anti-line 10 tumor activity in pre-immunized guinea pigs.

Correspondence and present address: C.R. Ahsan, Department of Microbiology, University of Dhaka, Dhaka 1000, Bangladesh

2. MATERIALS AND METHODS

2.1. *BCG and tumor cells*

Mycobacterium bovis BCG (Tokyo strain 172) was cultured on Ogawa's egg slants (Eiken Chemical Co., Tokyo) and inoculated into one liter of Dubos medium (Eiken Chemical Co., Tokyo) in five Erlenmeyer flasks and incubated at 37°C. After 2–3 weeks, the cells were separated by centrifugation at 16 000 × *g* for 25 min and washed in saline.

Diethylnitrosamine induced line 10 hepatocarcinoma cells [20] in strain 2 guinea pigs, were maintained in ascitic form by serial passages (6–10 passages) and the ascitic fluid was collected between 12–16 days after the intraperitoneal inoculation of the tumor cells. Viability of the tumor cells was checked by the Trypan blue dye exclusion method and the cells were washed in saline.

2.2. *Water extraction method*

The saline-washed BCG cells were suspended in 50 vols of distilled water. The suspension was mildly shaken (60 oscillations per min) for 8 h at 37°C and centrifuged at 16 000 × *g* for 30 min and the supernatant was re-centrifuged at 105 000 × *g* for 2 h. The supernatant after ultracentrifugation containing soluble water extracted materials was lyophilized.

The water extraction method for the line 10 tumor cells differed a little where the tumor cell suspension in distilled water (25 vols) was shaken for 1 h at 37°C [21].

2.3. *Transmission electron microscopy (TEM)*

This was performed to check the surface appearances of the BCG cells after water extraction, where samples on copper grids supported by collodion were negatively stained by 1% uranyl acetate and viewed under electron microscope at an accelerating voltage of 75 kV.

2.4. *Protein estimation*

Protein content of all samples was estimated by using the Sigma Micro Protein Determination Kit.

2.5. *Isolation of the BCG 64 kDa protein*

A 12% separating gel was made in combination with a 5% stacking gel [22] and approximately 300 μg of protein of the water extracted materials from BCG was applied to the gel. The separated proteins in

gels were then either stained with Coomassie brilliant blue R250 (Bio-Rad) or periodic acid Schiff's reagent, or transferred to nitrocellulose membranes [23]. The membrane was stained in 0.1% Ponceau red solution to visualize the proteins and the center of a 64 kDa band (BCG 64 kDa), as determined by the molecular weight standards, was cut out carefully, destained in phosphate buffered saline (PBS) and sonicated in 5 ml of PBS until the membrane reduced to a powder fine enough to pass through a 25-gauge hypodermic needle and was lyophilized [24]. The apparent molecular weight was estimated using the following proteins as standards: bovine albumin 66 kDa, egg albumin 45 kDa, glyceraldehyde-3-phosphate dehydrogenase 36 kDa, carbonic anhydrase 29 kDa, trypsinogen 24 kDa and trypsin inhibitor 20 kDa.

2.6. *Immunization*

Adult rabbits were immunized at one week intervals with three intramuscular injections of the sonicated membrane containing the BCG 64 kDa (approximately 50 μg of protein/kg body weight), suspended in saline. Titers of antibodies in serum were checked by the gel diffusion test and sera (Rabbit anti BCG kDa) were collected 6 days after the last injection and stored at -80°C.

2.7. *Immunoblotting*

Contamination of other proteins with the BCG 64 kDa was studied by immunoblotting [23]. Approximately 30 μg of protein of the water extracted materials from BCG was applied to a lane of SDS-PAGE and the separated proteins in the gel were transferred to a nitrocellulose membrane for immunoblotting analysis, where rabbit anti-BCG 64 kDa served as the 1st antibody and alkaline phosphatase conjugated swine anti-rabbit immunoglobulins (DAKOPATTS) as the 2nd antibody.

2.8. *Antigenic cross-reactivity*

Distribution of common antigenic determinant(s) between the BCG 64 kDa and line 10 tumor cells were studied by the indirect immunofluorescence techniques [19] and immunoblotting [23].

Tumor cells were fixed in absolute ethanol, washed with PBS and incubated with rabbit anti-BCG 64 kDa and fluorescein isothiocyanate (FITC) conjugated anti-rabbit IgG (Cappel Lab. Inc. USA) as 1st and 2nd antibodies, respectively. Tumor cells treated with non-immunized rabbit serum as 1st antibody served as control.

Approximately 30 μg of protein of the water extracted materials from BCG and line 10 tumor cells were applied to the SDS-PAGE and proceeded as described earlier (Immunoblotting).

2.9. *Anti-line 10 tumor activity*

Anti-line 10 tumor activity of the BCG 64 kDa was demonstrated in strain 2 guinea pigs, which were immunized three times with one-week intervals with the sonicated membrane containing BCG 64 kDa (approximately 50 μg of protein/kg body weight). The titer of antibodies in sera was checked by the gel diffusion test and all animals were challenged with approximately 1×10^6 live line 10 tumor cells suspended in RPMI 1640 medium, intradermally, 6 days after the last immunization. All animals were observed for 40 days for the growth of tumors and the association of BCG 64 kDa in the in vivo rejection or inhibition of live tumor cell challenge in the animals were statistically analyzed by the Student's *t*-test. Thirteen guinea pigs were included in the test group and 10 guinea pigs served as control, which were injected three times with one week intervals with fresh sonicated nitrocellulose membrane (same turbidity as test group).

3. RESULTS AND DISCUSSION

The water-extracted materials showed one major protein band of 64 kDa and some other low molecular weight proteins in SDS-PAGE after Coomassie brilliant blue R250 staining (Fig. 1). The major band was also faintly stainable in periodic acid Schiff's reagent, suggesting the nature of the 64 kDa band to be a protein with carbohydrate moieties. Isolation of BCG 64 kDa with less or no contamination of other proteins was demonstrated by immunoblot analysis, where only a single band was developed on the nitrocellulose membrane (Fig. 1).

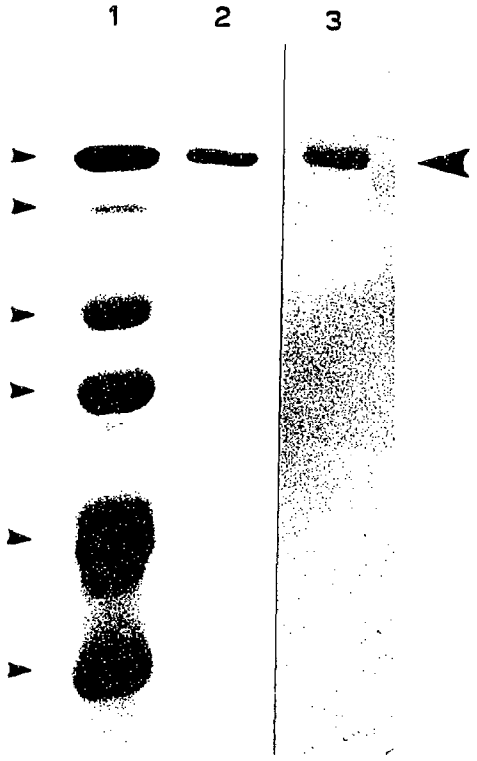

Fig. 1. Water-extracted materials from BCG showed one major protein band of 64 kDa in SDS-PAGE after Coomassie brilliant blue R250 staining (lane 2). No contamination of other proteins with BCG 64 kDa was demonstrated by immunoblot analysis (lane 3), where rabbit anti-BCG 64 kDa served as the 1st antibody. Lane 1 shows the molecular weight standards in kDa: bovine albumin 66, egg albumin 45, glyceraldehyde-3-phosphate dehydrogenase 36, carbonic anhydrase 29, trypsinogen 24, and trypsin inhibitor 20.

The simple low ionic strength extraction by the use of distilled water has been used previously to release several protein components from bacteria and other sources [21,25,26]. One main advantage of the water extraction, as has been revealed by electron microscopy, is that it only removed the surface components of the cells without significantly affecting the morphological structures of the cells (data not shown), thus making subsequent purification of the surface proteins easier. Isolation of the soluble 64 kDa protein from the water extracted materials also suggested that the BCG 64 kDa is a surface protein.

In recent studies, we described the major surface components of line 10 hepatoma cells and their antigenicities, where 5 proteins with carbohydrate moieties of apparent molecular weights of 44, 46, 62, 64 and 68 kDa were prominent [21], and suggested com-

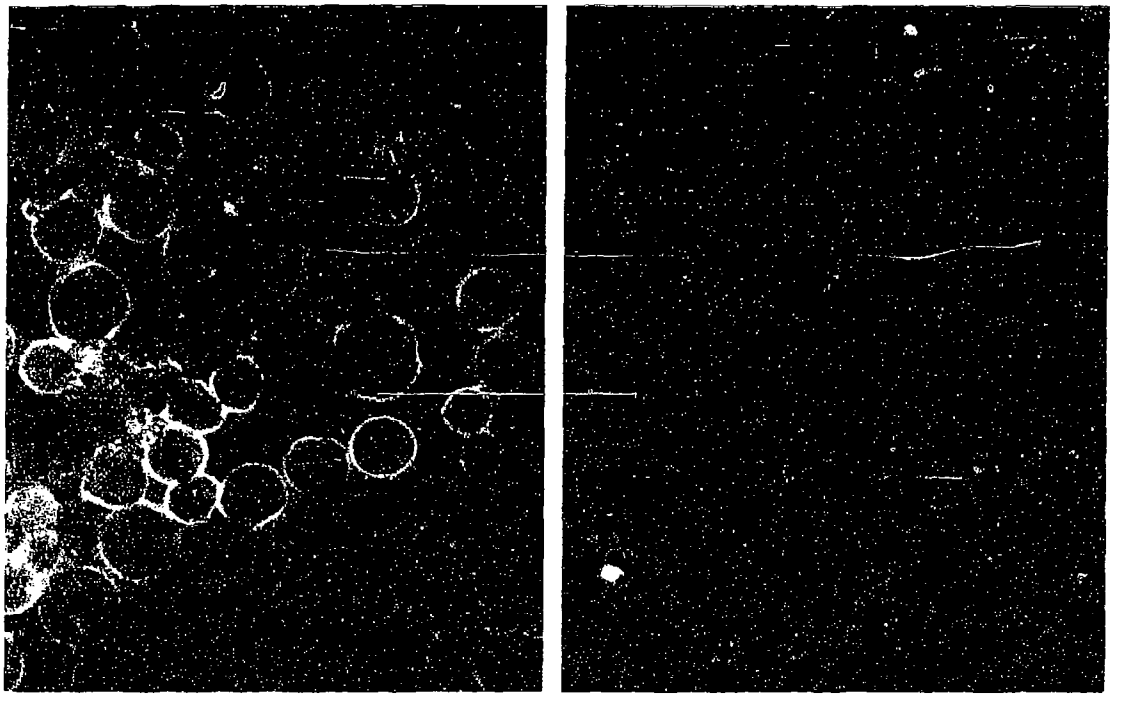

Fig. 2. Indirect immunofluorescence analysis showing sharp fluorescences from line 10 tumor cells treated with rabbit anti-BCG 64 kDa as the 1st antibody, suggesting that BCG 64 kDa and line 10 tumor cells have common antigenic determinant(s) (A, 800×). However, the tumor cells treated with non-immunized rabbit serum as 1st antibody did not show any fluorescence (B, 800×).

mon antigenic determinants between the surface extracts of line 10 tumor cells (and others) and BCG using BCG-monoclonal antibodies [19]. In this study, the indirect immunofluorescence analysis showed sharp fluorescences from line 10 tumor cells treated with rabbit anti-BCG 64 kDa (Fig. 2), indicating that the BCG 64 kDa protein and line 10 tumor cells have antigenic determinant(s) in common, which are probably present on the surfaces of the tumor cells. This is again supported by the immunoblot analysis where a faint but clear band from line 10 tumor water-extracted materials, corresponding to the BCG 64 kDa band, was obtained on nitrocellulose membranes (data not shown). All these data combined, confirm each other and clearly indicate that the antigenic determinants between the BCG 64 kDa and the 64 kDa major surface antigenic component of line 10 tumor cells are identical.

Strong antitumor activity of BCG 64 kDa was clearly demonstrated in the test animals when compared with the control group. The experiment was repeated to check the reproducibility of the results, and the test animals which received BCG 64 kDa showed either complete rejection or partial inhibition of the tumor growth when observed for 40 days after tumor transplantation (Fig. 3). However, the control animals which were injected with fresh sonicated membrane only, developed big tumors without much difference in size, suggesting that the nitrocellulose membrane only acted as an adjuvant [24] and did not affect the results of our experiments. Evidence that the isolated BCG 64 kDa can induce an immune response in normal guinea pigs is suggested by the fact that all immunized animals demonstrated significantly increased titers of anti-BCG 64 kDa antibody. However, the most relevant and simultaneously the most stringent criterion for the functional integrity of the isolated BCG 64 kDa antigen, is the ability to induce protective tumor immunity in nor-

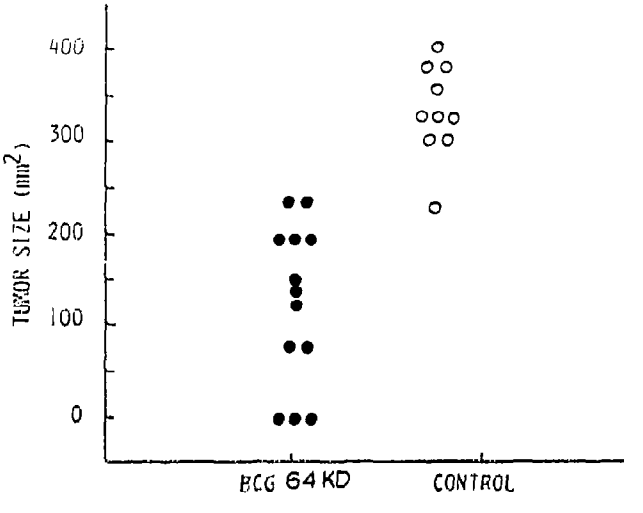

Fig. 3. The test guinea pigs which were immunized with the BCG 64 kDa, showed either complete rejection or partial inhibition of line 10 tumor growth when observed for 40 days after tumor cell challenge. However, all control animals developed big tumors without much difference in size after tumor cell challenge.

mal animals either by activating lymphocytes or by inducing Natural Killer (NK) cells by acting as an adjuvant, which eventually may inhibit the tumor cells. This criterion demands not only that BCG 64 kDa be immunogenic but also that the level and type of immunity that is developed provide the immunized animals with the ability to reject or inhibit a viable tumor cell challenge. The anti-line 10 tumor activity of BCG 64 kDa in the pre-immunized animals also suggests that BCG 64 kDa is probably identical with the tumor specific antigen.

Sharing of antigenic determinants between BCG and line 10 tumor cells has also been suggested by others. Boros and Rapp found that sera from guinea pigs immunized with BCG reacted with line 10 cells [16]. Minden and co-workers showed that the line 10 cells shared some determinants with BCG [17], and Bucana and Hanna used immunoelectronmicroscopy to demonstrate binding of anti-BCG to the surface of line 10 and human melanoma cells [27]. Our study confirms these earlier findings and additionally suggests that (i) antigenic determinants between the two 64 kDa surface proteins of BCG and line 10 hepatoma cells are common and (ii) that the 64 kDa protein from BCG is probably identical with the tumor specific antigen.

Although we are not sure, at this stage of the work, about the identity or similarity of our BCG 64 kDa protein and the BCG 64 kDa protein described by others [4-8], these data definitely provide us with a basis for the further characterization of the BCG 64 kDa protein as an agent for tumor diagnosis and therapy.

REFERENCES

[1] Gillis, T.P., Miller, R.A., Young, D.B., Khanolkar, S.R. and Buchanan, T.M. (1985) Infect. Immun. 49, 371-377.

[2] Buchanan, T.M., Nomaguchi, H., Andarson, D.C., Young, R.A., Gillis, T.P., Britton, W.J., Ivanyi, J., Kolk, A.H.J., Closs, O., Blool, B.R. and Mehra, V. (1987) Infect. Immun. 55, 1000-1003.

[3] Shinnick, T.M. (1987) J. Bacteriol. 169, 1080-1088.

[4] Shinnick, T.M., Vodkin, M.H. and Williams, J.C. (1988) Infect. Immun. 56, 446-451.

[5] Emmrich, F., Thole, J., van Embden, J. and Kaufmann, S.H.E. (1986) J. Exp. Med. 163, 1024-1029.

[6] Harboe, M., Nagai, S., Patarroyo, M.E., Torres, M.L., Ramirez, C. and Cruz, N. (1986) Infect. Immun. 52, 293-302.

[7] De Bruyn, J., Bosmans, R., Turner, M., Weckx, M., Nyabenda, J., Vooren, J.V., Falmagne, P., Wiker, H.G. and Harboe, M. (1987) Infect. Immun. 55, 245-252.

[8] Thole, J.E.R., Keulen, W.C., Kolk, A.H.J., Groothuis, D.G., Berwald, L.G., Tiesjema, R.H. and van Embden, J.D.A. (1987) Infect. Immun. 55, 1466-1475.

[9] Reif, A.E. and Kim, C.A.H. (1971) Cancer Res. 31, 1606-1612.

[10] Baldwin, R.W. and Pimm, M.V. (1973) Natl. Cancer Inst. Monogr. 39, 11-17.

[11] Pinsky, L., Hirshaut, Y. and Oettgen, H. (1973) Natl. Cancer Inst. Monogr. 39, 225-228.

[12] Baldwin, R.W. and Pimm, M.V. (1974) Br. J. Cancer 30, 473-476.

[13] Weiss, D.W., Bonhag, R.S. and Leslie, P. (1966) J. Exp. Med. 124, 1039-1065.

[14] Zbar, B., Ribi, E.E. and Rapp, H.J. (1973) Natl. Cancer Inst. Monogr. 39, 3-6.

[15] Azuma, I., Ribi, E.E., Meyer, T.J. and Zbar, B. (1974) J. Natl. Cancer Inst. 52, 95-102.

[16] Boros, T. and Rapp, H.J. (1973) J. Natl. Cancer Inst. 51, 1085-1086.

[17] Minden, P., McClatchy, J.K., Wainberg, M. and Weiss, D.W. (1974) J. Natl. Cancer Inst. 53, 1325-1331.

[18] Minden, P., Sharpton, T.R. and McClatchy, J.K. (1976) J. Immunol. 116, 1407-1414.

[19] Sasaki, J., Kitagawa, M., Tamagake, T., Narita, S., Ahsan, C.R. and Lu, C. (1989) Microbiol. Immunol. 33, 951-955.

[20] Argus, M.F. and Hoch-Ligeti, C. (1963) J. Natl. Cancer Inst. 30, 533-551.

[21] Ahsan, C.R. and Sasaki, J. (1989) Microbiol. Immunol. 33, 219-227.

[22] Laemmli, U.K. (1970) Nature 227, 680-685.

[23] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350-4354.

[24] Diano, M., Bivic, A.L. and Hirn, M. (1987) Anal. Biochem. 166, 224-229.

[25] Harris, J.R. (1971) Biochim. Biophys. Acta 229, 761-770.

[26] Oaks, E.V., Hale, T.L. and Formal, S.B. (1986) Infect. Immun. 53, 57-63.

[27] Bucana, C. and Hanna Jr, M.G. (1974) J. Natl. Cancer Inst. 53, 1313-1323.