SUMMARY

Antigenic Determinant(s) in Mycobacterium and Infected Macrophages.

Studies on the surface component(s) of mycobacteria and infected macrophages are of paramount importance in understanding the pathogenesis of the disease and can also help towards the identification of the targets for therapeutic interventions. Antigenic determinants on mycobacteria have been studied by using polyclonal antibodies, monoclonal antibodies and patient sera. These antigens have been linked with diagnosis, vaccine development and pathogenesis of the disease. The induction of mycobacterial genes during infection as well as altered expression of various adhesion molecules on infected cell surface has been demonstrated, but nothing much has been done to identify and characterize bacterial/host cell components specifically present on the infected cell.

Mycobacterium microti is a slow growing mycobacteria and belongs to *M. tuberculosis* complex. It resembles antigenically and taxonomically to *M. tuberculosis* and cause a disease similar to human tuberculosis in its natural host. It was once used as a vaccine and recently *M. microti* has also been isolated from immunocompromised patients. Accordingly, *M. microti* was chosen for the present study.

Taking malaria infected erythrocytes and leishmania infected macrophages as model systems for intracellular infections, recent studies from this lab have successfully demonstrated the identification of neo-antigenic determinants on infected cell surface using homologous antiserum, generated against infected cell membranes. In this study, this approach was extended to *M. microti* infected macrophages, with an objective to identify neo-antigen(s) associated with infected macrophages and those present on mycobacteria. For this, polyclonal antisera and panel of monoclonal antibodies (MAbs) were generated by immunizing heat killed bacteria (HKB), extract of *M. microti* (BE) and *M. microti* infected homologous macrophage (48h post infection) membranes (IMm). These antibodies were used to compare the antigenic profiles of mycobacteria and infected macrophages.

The titers of anti-HKB antiserum and anti-BE antiserum with their respective antigens were found to be 1:800 and 1:6400 respectively in ELISA. While, anti-IMm antiserum exhibited a titer of 1:200 with infected macrophages in IFA. When cross checked, anti-BE antiserum and anti-IMm antiserum reacted with heat killed bacteria, having a titer of 1:800 and 1600. While, anti-HKB antiserum and anti-IMm antiserum showed comparatively weaker bindings with bacterial extract having titers of 1:800 and 1:1600 respectively. In immunofluorescence assay, using heat fixed bacteria, all the three antisera reacted moderately.

Reactivity of various polyclonal antisera with infected cell was checked using immunofluorescence assay (IFA), flow cytometry and their binding sites were subsequently localized by immunoelectron microscopy. Anti-BE antiserum and anti-HKB antiserum did not react with infected cell surface. On the other hand, anti-IMm antiserum reacted with infected cell surface and this reactivity increased with the increase in post infection time. Antiserum raised against homologous normal macrophage membranes did not show any binding with infected cell.

Anti-IMm antiserum did not react with normal macrophages, cultured *in vitro* for 48h. Also it did not react with the surface of macrophages infected with either heat killed *M. microti* or with *M. tuberculosis* H37Ra. These results further established that the normal macrophage membrane associated components were neither immunogenic in homologous animals nor participated in binding with antisera and the reactivity of anti-IMm antiserum was specific to live *M. microti* infected macrophages.

With digitonin permeabilized *M. microti* infected macrophages, anti-HKB antiserum and anti-BE antiserum reacted with intracellular component(s) without showing any binding to cell surface in IFA. While, anti-IMm antiserum showed diffused fluorescence. Similar binding pattern was also observed by immunoelectronmicroscopy.

Antigenic components of *M. microti* recognized by various antisera, in immunoblotting, after SDS-PAGE separation under reducing conditions, were as follows :

i) Using bacterial extract, anti-BE antiserum reacted with proteins of molecular masses 105 kDa, 92 kDa, 87 kDa, 80 kDa, 73 kDa, 62- 67 kDa, 47 kDa, 45 kDa, 41 kDa, 38 kDa, 33 kDa, 25 kDa and <24 kDa and anti-HKB antiserum recognized 64 kDa and 48 kDa proteins. While, anti-IMm antiserum identified two antigens of molecular masses 50 kDa and 47 kDa, among these 50 kDa antigen was neither identified by anti-BE antiserum nor by anti-HKB antiserum.

ii) With cytosolic fraction, anti-BE antiserum identified 77 kDa, 72 kDa, 56 kDa, 50 kDa, 43 kDa, 40 kDa, 38 kDa, 33 kDa, 30 kDa , < 24kDa antigens and anti-HKB antiserum reacted with a protein of molecular mass 55 kDa. Whereas, anti-IMm antiserum did not react at all.

iii) With membrane fractions, 29 kDa, 24 kDa, < 24kDa and 29 kDa, 25 kDa antigens were recognized by anti-BE antiserum and anti-HKB antiserum respectively. Anti-IMm antiserum did not react with any of the membrane component of bacteria.

iv) In SDS treated cell wall fraction, only anti-BE antiserum identified the proteins of molecular masses 27 kDa, 23 kDa and a faint band at 48 kDa position.

In *M. microti* infected macrophages membrane, antigenic components were identified by immunoblotting. Anti-BE antiserum and anti-HKB antiserum did not react, whereas anti-IMm antiserum recognized a protein of molecular mass 63 kDa. None of the above antisera reacted with the membrane fraction of normal

macrophages, cultured *in vitro* for 48 h, thereby suggesting that 63 kDa antigen was exclusively present on infected macrophage membranes.

Cross reactivity of various polyclonal antisera with *M. tuberculosis* H37Ra and *M. smegmatis* was checked by ELISA. This was followed by the identification of cross reactive antigenic determinants by immunoblotting. In ELISA, anti-BE antiserum reacted strongly with *M. tuberculosis* H37Ra and *M. smegmatis* having titers of 1:3200 and 1:6400 respectively. Anti-HKB antiserum reacted with *M. tuberculosis* H37Ra as well as with *M. smegmatis* having a titer of 1:400. And 1:100.respectively. Whereas anti-IMm antiserum showed titers of 1:200 and 1:400 with *M. tuberculosis* H37Ra and *M. smegmatis* respectively.

In immunoblotting, using extract of *M. tuberculosis* H37Ra: anti-BE antiserum recognized proteins of molecular masses of 70 kDa, 68 kDa, 63 kDa, 55 kDa, 52 kDa, 46 kDa, 44 kDa, 41 kDa, 39 kDa, 36 kDa, 31 kDa, 30 kDa, 26 kDa antigens; anti-HKB antiserum identified 53 kDa and 41 kDa antigens, and anti-IMm antiserum recognized 40 kDa antigen. While using extract of *M. smegmatis*, anti-BE antiserum reacted with the proteins of molecular masses 62 kDa, 58 kDa, 44 kDa, 40 kDa and 35 kDa, whereas, anti-HKB antiserum and anti-IMm antiserum did not react. Thus, anti-BE antiserum identified a protein of molecular mass 41 kDa in *M. microti* and *M. tuberculosis* H37Ra and a protein of molecular mass 40 kDa in *M. microti* and *M. smegmatis*.

A panel of MAbs were generated using spleenocytes from mice immunized with either bacterial extract or with *M. microti* infected macrophage membranes. The observations based upon their reactivity pattern were :

i) In ELISA, MAbs B2B2, G8E3, C3G5 and C10B5 reacted with all the three preparations i.e heat killed bacteria, bacterial extract and cytosolic fraction of *M. microti*. MAbs C2B5, G10A4 and B2C8 reacted with bacterial extract and heat killed bacteria and MAbs D7B1, D4F1 and F1C2 reacted with specifically extract, cytosolic fraction and heat killed bacteria respectively.

ii) Using heat fixed mycobacteria, in IFA, MAbs G10A4, B2B2, C2B5, G8E3, C3G5 and C10B5 reacted strongly. Whereas, MAbs B2C8, D4F1 and F1C2 showed low reactivity and MAb D7B1 showed negligible reactivity.

iii) In immunoblotting with bacterial extract , antigens identified by MAbs B2B2, D7B1, D4F1 and G10A4 were of molecular masses 31 kDa, 19 kDa; 52 kDa, 29 kDa; 80 kDa, 51 kDa and 62 kDa, 30-31 kDa respectively. Two MAbs B2C8 and G8E3 recognized an antigen of similar molecular mass of 30 kDa. A streaking pattern along with prominent bands at either position 28 kDa or 50 kDa was identified by MAbs C2B5 and F1C2 respectively. While MAbs C3G5 and C10B5 showed streaking pattern without any discrete band.

iv) Proteins recognized in the extract by MAbs C2B5, G8E3, D7B1, C3G5 and C10B5 were also found to be present in the cytosolic fraction thus, indicating their cytosolic localization.

v) Two proteins of lower molecular masses (<24 kDa) were identified by MAbs G8E3 and C10B5 in SDS treated cell wall fraction of *M. microti*.

vi) MAbs C2B5, G10A4, C3G5 and C10B5 reacted with *M. microti* infected live macrophages (in IFA and flow cytometry). Similar observations were made in immunoelectronmicroscopy except for MAb G10A4 which did not react. None of the MAbs reacted with normal macrophages, *in vitro* cultured for 48 h.

vii) IFA with digitonin permeabilized, infected macrophages revealed that MAbs B2C8, B2B2 and D4F1 recognized intracellular antigens.

viii) With *M. tuberculosis* H37Ra in ELISA, MAbs B2C8 and C2B5 showed strong cross reactivity. MAbs B2B2, G8E3, C3G5 and C10B5 showed low reactivity. While, MAbs G10A4 and C10B5 exhibited comparatively low reactivity with *M. smegmatis*.

Number of cross reactive antigens in *M. tuberculosis* H37Ra and *M. smegmatis* were identified using MAbs. With extract of *M. tuberculosis* H37Ra,

MAbs G10A4, C2B5, C3G5, C10B5 and D4F1 reacted with proteins of molecular masses : 43 kDa; 61 kDa, 47 kDa; 27 kDa; 27 kDa; and 60 kDa, 44 kDa respectively.With extract of *M. smegmatis*, MAbs G10A4, C2B5, D7B1, F1C2 and B2C8 identified proteins of molecular masses : 64 kDa, 62 kDa; 65 kDa; 64 kDa, 62 kDa, 40 kDa; 62 kDa, 47-46 kDa; and 71 kDa, 65 kDa respectively. Thus, MAbs G10A4 and C2B5 reacted with all the three species.

Thus, important findings emanating from this study are: a) anti-infected macrophage membrane antiserum (IMm antiserum), unlike anti- bacterial antiserum (anti-BE antiserum) and anti-heat killed antiserum (anti-HKB antiserum) specifically reacted with *M.microti* infected macrophage surface and identified a 63 kDa antigen in infected macrophage membranes, b) few monoclonal antibodies (MAbs) reacted with infected macrophage membranes without recognizing normal macrophages, c) polyclonal antibodies and a panel of monoclonal antibodies identified a number of antigens present on *M.microti* and those cross reactive with *M tuberculosis* H37Ra and/or *M.smegmatis*. Such components may find wide application, not only clinically but also in understanding the mechanisms responsible for the intracellular survival of bacteria.