Membrane surface of *Mycobacterium microti*-infected macrophages antigenically differs from that of uninfected macrophages

Sadhana Majumdar a, Hardeep Kaur a, Harpreet Vohra b, Grish C. Varshney a, *

a Institute of Microbial Technology, Sector 39-A, Chandigarh 160036, India
b Department of Experimental Medicine, Post Graduate Institute of Medical Education and Research, Chandigarh 160016, India

Received 12 July 1999; received in revised form 6 December 1999; accepted 3 January 2000

Abstract

Identification of the antigenic changes in mycobacteria-infected macrophage may be important in understanding the mechanisms responsible for the intracellular survival of the bacteria. In the present study, *Mycobacterium microti*-infected macrophages were utilized to investigate the possibility of differentiating the infected cells from normal cells, based on the antigenic changes occurring in the membranes. Antisera were generated against bacterial extract, heat-killed bacteria and crude preparation of *M. microti*-infected homologous macrophage membrane. The reactivity of these antisera, towards in vitro infected macrophages, was compared by flow cytometry. Unlike anti-bacterial extract antiserum or anti-heat-killed bacterial antiserum, anti-infected macrophage membrane antiserum reacted with infected macrophage surface. This reactivity increased with the increase in post-infection time. However, it was not observed with uninfected macrophages, PMA- or lipopolysaccharide-activated macrophages and those harboring *Mycobacterium tuberculosis* H37Ra, heat-killed *M. microti* and *Leishmania donovani*. Interestingly, anti-infected macrophage membrane antiserum identified a 63-kDa antigen in *M. microti*-infected macrophage membranes which was not present in the membranes of normal macrophages, activated macrophages and those infected with *M. tuberculosis* H37Ra, heat-killed *M. microti* and *L. donovani*. Thus, membranes of *M. microti*-infected macrophages differ antigenically from those of the normal macrophages and infected homologous macrophage membrane antiserum provides a useful tool in studying such changes. © 2000 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Macrophage; Surface membrane; Antigenic change; Mycobacterium microti

1. Introduction

Like other intracellular pathogens, mycobacteria have developed a variety of mechanisms to survive within macrophages, the cells that are primarily meant for a major line of defense against invading pathogens [1,2]. Identification of mycobacterial products as well as alterations of host cell component in infected cells is of paramount importance in unraveling the mechanism responsible for the intracellular survival of mycobacteria.

Antigenic changes on the infected cell surface have been well characterized in parasitic [3–5] and bacterial infections [6,7]. Few reports have demonstrated the induction of mycobacterial genes during infection [8,9] and an altered expression of various known adhesion and costimulatory molecules on infected cell surface [10,11]. Not much has been done to identify and characterize the determinant(s) specifically present on *Mycobacteria*-infected cells.

Our group has focused mainly on parasite surface and infected cell surface for: (i) identification of neo-antigenic determinant(s) and, (ii) site specific drug delivery. We first showed that polyclonal antiserum and monoclonal antibodies, generated by immunization with parasite-infected homologous cell membranes, react with the neo-antigenic determinants on the infected cell surface both in vitro and in vivo [12,13]. This was followed by a demonstration that such an immunization strategy is useful in diverting the immune response towards clinically relevant ‘minor’ antigenic determinants in the absence of major parasite immunogenic components [14]. We now wished to investigate whether such strategy can provide a tool to differentiate *Mycobacterium microti*-infected cell surface from that of normal macrophages.
2. Materials and methods

2.1. Bacteria and animals

*M. microti* was obtained from Tuberculosis Research Center, Madras, India, and maintained on Lowenstein–Jensen medium (Difco, USA). For experimental purposes, cells were grown at 37°C in Middle Brook 7H9 medium containing 0.05% Tween-80 and harvested at the exponential growth phase. Colony forming units were counted by serial dilution on 7H9 agar plates. Bacteria were preserved in 1-ml aliquots at −70°C.

Inbred BALB/c mice (6–8 weeks old) were used in the study. These animals, procured from Jackson Laboratory, Bar Harbor, USA, were reared in the central animal facility of the institute.

2.2. In vitro infection of peritoneal macrophages

Peritoneal macrophages were harvested from mice that had received, 5 days before, an intraperitoneal injection of 2 ml of Brewer’s thioglycollate broth. Cells collected from peritoneal fluid by centrifugation (250 × g, 10 min) were suspended in RPMI 1640 medium (Gibco, USA) containing 10% fetal calf serum and then dispensed in each 90-mm petri-dish (3 × 10^7 cells per dish). After 1 h of incubation at 37°C, non-adherent cells were washed off and the adherent cells were incubated at 37°C for 24 h. Monolayers were infected with *M. microti* or *Mycobacterium tuberculosis* H37Ra (10 bacteria per macrophage) for 4 h, extracellular bacteria were washed off with phosphate-buffered saline (PBS) and infected cells were further incubated for 48 h. For in vitro infection of peritoneal macrophages with *Leishmania donovani* promastigotes, the method followed was essentially the same as described in our earlier paper [13].

2.3. Activation of macrophages with PMA or lipopolysaccharide (LPS)

Macrophage monolayers were activated with either PMA or LPS following a published protocol [15]. For this, the cells were treated with PMA (0.1 μg ml^-1) or LPS (2 μg ml^-1) for 48 h. Activation was verified by measuring reactive oxygen intermediate or reactive nitrogen intermediate production [16].

2.4. Preparation of membrane fractions

Crude membrane fractions of normal macrophages, activated macrophages and infected macrophages (cultured in vitro for 48 h) were prepared according to the method.

---

![Fig. 1. Flow cytometric analysis of M. microti-infected macrophages showing binding with: (a) FITC-conjugated secondary antibody, (b) pre-immune serum, (c) anti-NMm antiserum, (d) anti-HKB antiserum, (e) anti-BE antiserum, (f) anti-IMm antiserum and (g) anti-MAC-1β monoclonal antibody (TIB 218 cell supernatant) which was taken as a positive control. Values shown in the parentheses are the MFI scores.](https://academic.oup.com/femspd/article-abstract/28/1/71/486436)
described [17] with little modifications [13]. Briefly, macrophages were collected from petri-dishes by scraping followed by centrifugation. Cells were suspended in homogenizing buffer containing 10 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 1 mM EDTA and 0.5% PMSF and then subjected to three cycles of freezing at −70°C and thawing at 37°C. Disrupted cells were homogenized and then centrifuged at 600 × g for 20 min to remove contaminating organelles. Supernatant was further fractionated by ultracentrifugation (100,000 × g, 1 h, 4°C) and the pellet, rich in cellular membranes, was used for immunization.

2.5. Generation of polyclonal antisera

Mice (homologous) were immunized intraperitoneally with crude membrane fractions from *M. microti*-infected macrophages or normal peritoneal macrophages (from 2–3 × 10⁷ cells per animal) cultured in vitro for 48 h. Boosters were given every 21 days and after 3–4 boosters, antisera were prepared, pooled, decomplemented and stored at −70°C. Antisera were also generated in a similar manner against heat-killed (60°C, 60 min) bacteria (1–2 × 10⁷ bacteria per animal) [10] or against bacterial extract (50–100 μg per animal), prepared by homogenizing liquid N₂ frozen pellet of the bacteria in presence of glass beads followed by centrifugation (12000 × g, 20 min) [18]. Sera from pre-immunized animals were also taken as control. The reactivity of test sera was initially checked by immunofluorescence assay using air-dried and digitonin-permeabilized [19] infected macrophages. Anti-heat-killed bacterial antiserum (anti-HKB antiserum) and anti-bacterial extract antiserum (anti-BE antiserum) reacted well with intracellular bacteria, while anti-*M. microti*-infected macrophage membrane antiserum (anti-IMm antiserum) primarily reacted with infected cell membranes (data not shown).

2.6. Flow cytometry

Live infected macrophages after 48 h or required time periods of infection were taken for flow cytometric analysis that was carried out as described earlier [13]. After blocking of Fc receptors by normal rabbit immunoglobulins (100 μg ml⁻¹), for 1 h at 4°C, cells were reacted at 4°C for 1 h in each step with test or control sera (1:200 in PBS) followed by FITC-conjugated rabbit anti-mouse antibody. Samples were fixed in 0.5% paraformaldehyde and analyzed using exponential amplifiers on a LYSIS II software of FACScan (Becton Dickinson, USA). 10,000 cells were acquired after ‘live’ gating on exponential FSC/SSC parameter and the fluorescence was measured on gated cells only. The analysis of mean fluorescence intensity (MFI) was done on histograms where abscissa and ordinate denote exponential FITC fluorescence and relative cell counts, respectively.

2.7. Western blotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of membrane preparations was carried out on a 10% polyacrylamide separating gel and a 4% stacking gel under reducing conditions. Western blotting was performed at room temperature according to the published protocol [20]. After electrophoresis, proteins were transferred to a
3. Results

3.1. Recognition of M. microti-infected macrophage surface

Reactivity of various antisera towards live infected macrophage surface was compared by flow cytometry. The MFI values in Fig. 1 clearly show that only anti-IMm antiserum reacted (MFI 51) with live infected macrophage surface. While anti-HKB antiserum, anti-BE antiserum and control sera (pre-immune serum or anti-normal macrophage membrane antiserum (anti-NMm antiserum)) did not react (MFI < 13) with infected cell surface.

Fig. 2 shows the post-infection time dependence on infected macrophage surface binding of anti-IMm antiserum. No binding was observed at 4 h post-infection while the levels of binding increased with the increase in post-infection time period as checked up to 72 h.

3.2. Specificity of anti-infected macrophage membrane antiserum

Specificity of the anti-IMm antiserum was tested by checking its cross reactivity with normal macrophages, PMA- or LPS-stimulated macrophages and those harboring live M. tuberculosis H37Ra, heat-killed M. microti and live L. donovani. Results of flow cytometric analysis in Table 1 demonstrated that the reactivity of anti-IMm antiserum was confined to the live M. microti-infected macrophage surface only. While normal macrophages, activated macrophages and those infected with other pathogens did not react with the antiserum.

3.3. Identification of membrane component by immunoblotting

Reactivity of various antisera was first checked by immunoblotting, with the membrane fractions of M. microti-infected and normal macrophages, in vitro cultured for 48 h. Fig. 3 shows that anti-IMm antiserum recognized an antigen of an apparent molecular mass of 63 kDa in infected macrophage membranes, without reacting with normal macrophage membranes. While anti-HKB antiserum, anti-BE antiserum and control serum did not show any binding with either infected macrophage membranes or normal macrophage membranes.

In order to further check the specificity of 63-kDa protein, immunoblotting was also performed using anti-IMm antiserum with the membrane preparations from PMA- or LPS-activated macrophages and from those infected with live M. tuberculosis H37Ra or L. donovani. Results shown in Fig. 4 clearly indicated that the 63-kDa protein was not present in the membranes of either activated macrophages or infected with other intracellular pathogens.

Table 1

<table>
<thead>
<tr>
<th>Treatment or infecting agent</th>
<th>MFI valuesa</th>
<th>Normal mouse serum</th>
<th>Anti-IMm antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>21.96</td>
<td>31.62</td>
<td></td>
</tr>
<tr>
<td>+PMA</td>
<td>28.12</td>
<td>16.21</td>
<td></td>
</tr>
<tr>
<td>+LPS</td>
<td>30.73</td>
<td>14.10</td>
<td></td>
</tr>
<tr>
<td>+M. microti (live)</td>
<td>19.23</td>
<td>90.46</td>
<td></td>
</tr>
<tr>
<td>Experiment B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>21.03</td>
<td>22.03</td>
<td></td>
</tr>
<tr>
<td>M. tuberculosis H37Ra (live)</td>
<td>18.41</td>
<td>27.32</td>
<td></td>
</tr>
<tr>
<td>M. microti (heat-killed)</td>
<td>34.54</td>
<td>37.04</td>
<td></td>
</tr>
<tr>
<td>M. microti (live)</td>
<td>10.86</td>
<td>101.33</td>
<td></td>
</tr>
<tr>
<td>L. donovani (live)b</td>
<td>17.18</td>
<td>15.71</td>
<td></td>
</tr>
</tbody>
</table>

a Normal macrophages, PMA- or LPS- activated macrophages and infected macrophages were cultured in vitro for 48 h prior to testing the binding.

b MFI value obtained using anti-L. donovani-infected macrophage membrane antiserum [13] with corresponding infected cell surface was 83.92.

Values are representative of three identical experiments.
4. Discussion

In this study, we describe for the first time the antigenic differences in the surface membranes of macrophages infected with *M. microti* and of normal macrophages using antibodies raised against membrane of the homologous macrophages infected with *M. microti*. We have identified a 63-kDa antigen in the membranes of infected macrophages. The antisera were generated against heat-killed bacteria, bacterial extract and crude membrane preparation from homologous macrophages infected with *M. microti*. The reactivity of antisera was compared towards infected macrophages and normal macrophages by flow cytometry and immunoblotting.

Using polyclonal antibodies, pathogen-induced antigenic changes on the infected cell surface have been demonstrated in a number of intracellular infections [3–7]. In malaria-infected erythrocytes, these changes include parasite-derived antigens on the infected cell surface [4] and modified host proteins [5]. Recently, we have shown that the homologous antiserum, generated against parasite-infected cell membranes, specifically reacted with the infected cell surface [13] as well as with clinically relevant antigen(s) on parasite cell surface [14]. The results in this study demonstrate for the first time that the technique works well in identifying antigenic changes in *M. microti*-infected macrophages. Unlike anti-HKB or anti-BE antisera, anti-IMm antiserum specifically recognized the infected cell surface. It has been pointed in earlier studies that the reactivity of the antibodies with the infected cell surface might result from: (i) non-specific interaction of a certain parasite antigen with the uninfected macrophages in the culture of in vitro infected macrophages [3,21], (ii) uptake and processing of soluble parasite antigen(s) by normal macrophages [22] and, (iii) due to adsorption via Fc receptors [23]. In this study, these were ruled out by the observation that both the uninfected macrophages in the infected cell population (data not shown) and normal macrophages, cultured in vitro for 48 h, were not recognized by anti-IMm antiserum. While non-specific adsorption via Fc receptors was taken care by blocking the Fc receptors prior to binding of the antiserum. Also, anti-normal macrophage (cultured in vitro for 48 h) membrane antiserum did not react with either normal or infected macrophage membranes. This further confirmed our earlier observation that normal macrophage components are not immunogenic in homologous animals [13].

In *Leishmania*-infected macrophages, the parasite antigen on the infected cell surface has been demonstrated either during parasite attachment and internalization [24] or later with increase in post-infection time period [25]. This study lacks the experimentation in depth to determine the mechanism(s) responsible for the observed antigenic changes in *M. microti*-infected macrophages. Although, non-reactivity of anti-IMm antiserum with PMA-stimulated or LPS-stimulated macrophages indicated that macrophage activation markers were not involved. Anti-IMm antiserum from different batches reacted similarly with the
infected macrophage membranes. However, some batch to batch variation was observed in its reactivity towards bacterial lysate, which was either negligible or at low levels (data not shown). Nevertheless, increased surface reactivity of anti-IMm antiserum with infected macrophages with the increase in post-infection time pointed out the intracellular origin of the antigenic determinant(s) responsible for surface reactivity. The possible explanations for this are: (i) the involvement of mycobacterial genes expressed under an intracellular hostile environment of macrophages, including those for stress proteins [8,9,26] and/or, (ii) the existence of a similar mechanism as proposed for Leishmania-infected macrophages, i.e. incorporation of reactive epitope into the phagolysosome membranes, followed by subsequent fusion of that membrane with the macrophage surface membranes [3].

The 65-kDa heat shock protein of Mycobacteria appears to be a major immunoreactive protein during the course of tuberculosis infection and following vaccination with BCG [27]. The presence of 63-kDa antigen in the infected macrophage membranes, identified in this report, does not seem to resemble with 65-kDa protein since monoclonal antibody HAT5 (anti-65-kDa antibody) did not react with it in immunoblotting (data not shown). It is also unlikely that the antigen is expressed along with MHC since: (i) it is a very well established fact that only peptides of nearly 20 amino acids get associated with MHC molecules. However, our protein is of high molecular mass, (ii) down regulation of MHC molecules has been reported after M. microti infection [28] and, (iii) to the best of our knowledge, no report has yet been published demonstrating the generation of antibodies against MHC-peptide complex. It may be worth mentioning that this study further substantiates our previous observation [13], successfully demonstrating the generation of antibodies against the infected macrophage membranes. Further, 63-kDa antigen appears to be specifically expressed on the membranes of M. microti-infected macrophages since it was not present on the membranes of either activated macrophages or infected with other intracellular pathogens. Interestingly, antisera generated in a similar fashion against L. donovani-infected macrophage membranes contained antibodies specific to the parasite strain used for infection [14]. Thus, the technique might have wide application in identifying the pathogen specific antigens. Studies are underway to generate monoclonal antibodies to characterize 63-kDa antigen, which might help in elucidating the mechanism(s) responsible for the intracellular survival of mycobacteria.

Acknowledgements

This work was supported by grants from the Council of Scientific and Industrial Research (CSIR), India. We thank Dr. Javed Agrewala for the critical reading of the manuscript. The research fellowship, granted to S.M. by CSIR, is gratefully acknowledged. This is Institute of Microbial Technology communication 008/99.

References