Use of the skin protection assay in experimental syphilis to assess protective immunity against a specific Treponema pallidum surface epitope

David R. Blanco *, Cheryl I. Champion, Michael A. Lovett

Department of Medicine, Division of Infectious Diseases, University of California at Los Angeles, Los Angeles, CA 90095, USA

Received 26 April 2005; received in revised form 2 June 2005; accepted 7 June 2005

First published online 23 June 2005

Edited by J.-I. Flock

Abstract

We have recently shown that a monoclonal antibody, designated M131, that binds a surface phosphorylcholine epitope on Treponema pallidum possesses complement-dependent killing activity and confers partial protection in rabbits following passive immunization (Blanco et al., 2005, Infect. Immun. 73:3083–3095). In this study, the protective potential of M131 was further tested using the rabbit skin protection assay of Titus and Weiser. Both M131 and infection-derived immune rabbit serum resulted in significant lesion delays corresponding to at least a 90% reduction of the treponemal challenge inoculum. The skin protection assay provides a way to assess the protective potential of specific immunogens while using far less antibody than in passive immunization protocols.

Keywords: Treponema pallidum; Skin protection assay; NRS:, normal rabbit serum; IRS:, immune rabbit serum

1. Introduction

Syphilis is a multi-staged chronic infection caused by the spirochete Treponema pallidum subsp. pallidum (T. pallidum). The clearance of T. pallidum from the initial lesion in experimental rabbit syphilis has been associated with opsonizing antibody [1–3] whereas the subsequent establishment of immunity to challenge reinfection has been correlated with high titered complement-dependent bactericidal antibody [4,5]. Passive immunization of rabbits with infection-derived immune rabbit serum (IRS) confers significant partial protective immunity [6–9]. These findings have indicated that specific antibody plays a key role in the protective immune response that develops during syphilitic infection.

Over the past 20 years of syphilis research much interest has been directed toward the characterization of the T. pallidum outer membrane and its protein constituents in an effort to identify surface targets of bactericidal antibody and protective immunity. The outer membrane of T. pallidum possesses several unique features including the absence of lipopolysaccharide [10–12], a much higher lipid:protein ratio as compared to typical gram negative bacteria [13], a low density of membrane spanning protein [14,15], and a small number and amount of membrane associated lipoprotein [16].

Recently, there has been interest in the T. pallidum repeat (tpr) gene family due to their potential for antigenic variation and homology with the major surface proteins.
of *T. denticola* [12,17–19]. One of these proteins, TprK, has been reported to be a target of opsonic antibody and elicit partial protection in rabbits [19,20], although these findings were subsequently challenged [21]. Physical localization on the surface of *T. pallidum* has not been demonstrated for any of the previously identified outer membrane or Tpr proteins, nor has bactericidal antibody been elicited by immunization with native or recombinant forms. It has been hypothesized that the *T. pallidum* outer membrane lacks immunogenic surface exposed proteins [22].

We previously showed that mouse immunization with purified *T. pallidum* outer membrane vesicles (OMV) elicits a level of complement dependent bactericidal activity that is over 30 times greater than that of IRS [23]. OMV immunization of mice resulted in the isolation of a monoclonal antibody, termed M131, with potent complement dependent killing activity and which conferred significant partial protection in rabbits following passive immunization [24]. The physical binding of M131 to the surface of *T. pallidum* was demonstrated by both immunoelectron microscopy and immunofluorescence using the agarose gel microdroplet assay [25]. The specific target of M131 was found to be membranous phosphorylcholine containing lipid rather than a protein [24].

In this study, we have further tested the protective immunity potential of M131 by using the rabbit skin protection assay developed by Titus and Weiser [6]. This assay was first used over 25 years ago to test the protective immune potential of IRS and IgG isolated from IRS. The assay affords the advantage over conventional passive immunization by using relatively small amounts of serum to achieve passive protection. Thus a goal of this study was to determine if a comparable level of protection, as we have recently reported for M131 using conventional passive immunization, could be achieved using the skin protection assay.

### 2. Materials and methods

#### 2.1. Source of *T. pallidum*

*T. pallidum* subsp. *pallidum*, Nichols strain, was maintained by testicular passage in New Zealand White rabbits as described previously [26]. *T. pallidum* used for experiments was extracted from infected rabbit testicles in phosphate-buffered saline, pH 7.4 (PBS). Immune rabbit serum (IRS) was acquired from rabbits infected intratesticularly for a period of 6 months and shown to be immune to symptomatic infection following challenge at intradermal sites with $1 \times 10^3$ *T. pallidum*. Normal rabbit serum (NRS) was acquired from animals with non-reactive venereal disease research laboratory (VDRL) serology [27].

#### 2.2. Rabbit skin protection assay

M131 was tested for protective immunity in rabbits using the rabbit skin protection assay described by Titus and Weiser [6]. *T. pallidum* was extracted from infected rabbit testes, as described above, and resuspended into heat-inactivated (56 °C/30 min) normal rabbit serum (H-NRS) or heat-inactivated immune rabbit serum (H-IRS) to a concentration of $1 \times 10^4$ organisms/ml. To additional treponemal H-NRS suspensions, M131 or TEPC-183 (Sigma, St. Louis, MO), an irrelevant IgM monoclonal antibody, was added to a final concentration of 3 mg/ml. All suspensions were incubated for no longer than 30 min at room temperature in the inoculating syringes prior to challenge. In the absence of complement, *T. pallidum* remain motile and viable for up to 16 h of in vitro incubation [28]. For suspensions containing NRS or IRS, each of two rabbits with non-reactive VDRL serology were challenged intradermally on their shaved backs with 100 μL of the suspension at 4–8 sites per rabbit ($1 \times 10^3$ organisms/site). For suspensions containing TEPC-183 or M131, each of three rabbits with non-reactive VDRL serology were challenged intradermally with 100 μL of the suspension at 8 sites per rabbit ($1 \times 10^3$ organisms/site). All animals were observed daily for lesion appearance and development. Significant differences of mean lesion incubation periods were compared by the two-tailed t-test analysis.

### 3. Results and discussion

In this study, we used the skin protection assay developed by Titus and Weiser [6] to provide further confirmation of the protective immunity potential of monoclonal M131 and to determine whether this assay, which uses relatively small amounts of antibody, can be used as a substitute for conventional passive immunization. In the skin protection assay, virulent *T. pallidum*

<table>
<thead>
<tr>
<th>Table 1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Syphilitic lesion development in rabbits following intradermal challenge of <em>T. pallidum</em> admixtures</strong></td>
</tr>
<tr>
<td>Admixture</td>
</tr>
<tr>
<td>-----------</td>
</tr>
<tr>
<td>NRS</td>
</tr>
<tr>
<td>IRS</td>
</tr>
<tr>
<td>TEPC-183 MAb</td>
</tr>
<tr>
<td>M131 MAb</td>
</tr>
</tbody>
</table>

<sup>a</sup> Groups of 2–3 rabbits were challenged with admixtures containing *T. pallidum* and the indicated serum or monoclonal antibody. Each animal was challenged intradermally with a total of $1 \times 10^3$ *T. pallidum* at each of 4–8 sites.

<sup>b</sup> Range represents the day to first detection of erythematous and indurated lesions.

<sup>SE</sup>: standard error.

<sup>a</sup> $P < 0.001$, compared with the results of the control MAb and NRS.
suspensions are combined with antibody, in the absence of complement, and immediately inoculated intradermally into rabbits. The killing of *T. pallidum* following inoculation is believed to result from a bactericidal mechanism by the contribution of endogenous complement and/or by the effects of opsonization and professional phagocytes.

As shown in Table 1, all sites inoculated with admixtures of normal rabbit serum (NRS) (12 sites) and TEPC-183 control MAb (24 sites) showed a mean time to the appearance of syphilitic lesions of 11 ± 0 (±standard error) days post-challenge. By comparison, significant delays in the appearance of lesions occurred at sites inoculated with admixtures of IRS (12 of 12 sites) and M131 (23 of 24 sites) showing a mean time of 18.8 ± 0.83 and 16.1 ± 0.37 days, respectively (Table 1 and Fig. 1). These significant delays in lesion appearance of 7.8 and 5.1 days for admixture inoculations of IRS and M131, respectively, correspond to at least a 90% reduction in the number of virulent organisms following inoculation based upon previous inoculation control studies [8,29,30].

---

Fig. 1. Syphilitic lesion development 19 days after intradermal challenge with *T. pallidum* admixtures containing either normal rabbit serum (NRS), infection-derived immune rabbit serum (IRS), control TEPC-183 monoclonal antibody (CMAb), or monoclonal antibody M131 (M131). Injections sites are located adjacent to the black pen marks made on the skin. Inoculated sites with admixtures of IRS or M131 show delayed atypical lesions as compared to the control sites inoculated with admixtures of NRS or CMAb.
The meaning of lesion delays following intradermal challenge has been previously considered and is significant. Studies determining the relationship between *T. pallidum* inoculum size and incubation time [29,31] have estimated an in vivo generation time of 30–33 h for *T. pallidum*. Turner et al. showed that a 10-fold difference in inoculum size resulted in a four day difference in the incubation period [29]. In another study, a three day delay in lesion appearance was interpreted to represent a 10-fold reduction in the numbers of *T. pallidum* inoculated [8]. Izzat et al. [30] also explored the relationship between treponemal numbers and time to lesion appearance. The results showed that a two log difference in treponemal numbers injected resulted in a 3 day difference in time of lesion appearance when inocula of $10^2$ and $10^5$ treponemes were compared. A 3 day difference in time of lesion appearance was also found when inocula of $10^2$ and $10^5$ treponemes were compared. More recently, we have used real time PCR (RT-PCR) to quantify the number of treponemes over the course of lesion development in control lesions that develop normally as compared to lesions delayed in appearance following passive immunization using IRS and M131 [32]. As compared to control lesions, RT-PCR showed at least a 3-log decrease in the *T. pallidum* DNA copy number in lesions delayed up to 8 days in appearance. Thus, the RT-PCR findings taken together with the classic inoculation studies described above indicate that the 5.1 and 7.8 day delays in lesion appearance in this study, following admixture inoculations of M131 and IRS, respectively, represents at least a 90% reduction of the *T. pallidum* challenge inoculum in these rabbits. This finding is similar to our recent study where lesion delays following passive immunization with M131 were determined to represent at least a 99% reduction of the *T. pallidum* challenge inoculum [24]. These results demonstrate that the use of the skin protection assay, which uses relatively small amounts of antibody, represents a viable alternative to the large amounts of antibody required for conventional passive immunization to assess protective immunity.

The finding that M131 conferred a comparable level of protection to that of IRS is significant. IRS has been shown to have potent complement-dependent bactericidal activity [4,26,28], enhances phagocytosis of treponemes by macrophages [1,3], inhibits the attachment of *T. pallidum* to monolayer cell cultures [33–36], and confers significant partial protection from experimental disease by passive immunization [7–9]. Given these biological effects of IRS, it has long been believed that specific antibody plays a key role in challenge immunity that develops during the course of syphilitic infection. The comparable protection to IRS afforded by M131 in this study suggests that the phosphorylcholine surface target of M131 may represent a key protective immunogen. We are currently studying the M131 defined target to serve as an immunizing protective vaccine.

Acknowledgements

We thank Xiao-Yang Wu for his excellent technical assistance in these studies.

This work was supported by US Public Health Service grants AI21352 and AI-12601 to M.A. Lovett.

References


