Identification of a Novel Antigen from *Corynebacterium pseudotuberculosis* That Protects Sheep against Caseous Lymphadenitis

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A 40-kDa protein antigen from *Corynebacterium pseudotuberculosis* has been identified by application of a strategy that employs locally derived antibody-secreting cells (ASC). ASC probes generated by culture of ASC obtained from the lymph node draining the site of infection showed a specificity restricted to a 40-kDa antigen. Analysis of immunoblots with sequential serum samples taken from sheep during the course of experimental primary infection with *C. pseudotuberculosis* also revealed the 40-kDa antigen as an early immunodominant antigen. Sheep vaccinated with two 100-μg doses of a 40-kDa antigen preparation in aluminium hydroxide adjuvant were protected against infection with *C. pseudotuberculosis*, with an 82% reduction in the proportion of infected sheep and a 98% reduction in lung lesions. Sera from vaccinated sheep exhibited a strong response only to the 40-kDa antigen on immunoblots. These results strongly suggest that the 40-kDa antigen plays a major role in immunity to caseous lymphadenitis.

One of the major difficulties limiting the development of new defined-subunit vaccines is the identification or selection of protective antigens. Bacteria and parasites present the researcher and the host with a complex array of immunogenic molecules. To date, candidate vaccine antigens have been selected on the basis of their known or putative role in infection, e.g., bacterial toxins or essential enzymes of parasites such as glutathione-S-transferase (19), or on the basis of their identification with antibodies from immune sera. However, the complexity of the immune response to the pathogen is usually indicated by the very diverse antibody response in immune sera, and reliance on selection of molecules judged to be immunodominant on the basis of their reactivity with serum antibody has not always been successful in identifying protective antigens. For example, immunodominant membrane proteins of *Mycoplasma hyopneumoniae* (21) and *Plasmodium falciparum* (20) have not proved to be protective.

Caseous lymphadenitis (CLA) is a common and economically important disease of sheep and goats, caused by infection with *Corynebacterium pseudotuberculosis*. The disease is characterized by the formation of encapsulated necrotic or caseous lesions in tissue that in sheep are located primarily in peripheral lymph nodes and the lungs. *C. pseudotuberculosis* is a facultative intracellular pathogen that multiplies within macrophages. It is readily phagocytosed by nonimmune macrophages, and following phagocytosis with ovine (11) and caprine (22) macrophages, phagosomes fuse with lysosomes but bacteria continue to multiply within phagolysosomes, leading to cell death, release of bacteria, and ultimately a necrotic lesion. The response of the host is to contain the pyogranuloma by the formation of a strong collagen capsule (15). The immune response to natural infection has been shown to include a strong humoral component, with circulating antibodies detected to a large number of antigens (9). Cell-mediated immunity has also been implicated in control of CLA lesion development, with lymphocytes observed inside the collagen capsule in intimate contact with necrotic tissue (23). The intracapsular lymphocytes have a defined architecture of three layers, with high proportions of CD8 and of γδ T-cell subpopulations being striking features of the observed cellular response to *C. pseudotuberculosis* infection.

Two major virulence determinants of *C. pseudotuberculosis* are currently recognized—a potent phospholipase D (PLD) (14) of 31 kDa, believed important in facilitating spread of the disease, and a toxic lipid associated with the cell wall (4, 12). Production of both of these factors by strains of *C. pseudotuberculosis* has been correlated with virulence (17).

Vaccination against CLA in sheep has been attempted with a variety of antigens, with variable success. Jolly (14) showed the involvement of toxoid in protection. More recently, purified toxoid has been shown to provide a similar level of protection to a complex array of culture supernatant-derived antigens (5). Comparison of protection against CLA achieved with whole-culture, culture supernatant, and whole-cell antigens has led others (2, 3) to conclude that cell-associated antigens were required as well as toxoid for optimal protection. The ability of cell-associated antigens to protect lambs against CLA has been recently confirmed by using bacterial cell walls with muramyl dipeptide as an adjuvant (1). Hodgson et al. (13) have shown that infection of sheep with an avirulent toxin-minus strain of *C. pseudotuberculosis* with a deletion of the PLD toxin gene induces strong immunity to CLA, providing incontrovertible evidence that antigens other than PLD contribute to protection.

Despite many studies demonstrating the involvement of an antigen(s) other than toxoid in protection, no such antigen has been described. We report here the identification of an antigen from *C. pseudotuberculosis* by application of a strategy that utilizes the local immune response. Others have used B cells from a site of infection to derive monoclonal antibodies for antigen identification (24) but did not analyze directly the spectrum of specificities of antibodies produced by the B cells. Studies in our laboratories of the local immune response in
tissue at the time of infection, or rejection of infection, have shown that antibody-secreting cells (ASC) obtained locally produce antibodies of highly restricted specificity, especially compared with the serum response (16). Such ASC probes have been applied to the identification of a novel antigen, and furthermore, we demonstrate that vaccination of sheep with the native antigen provides a high level of protection against experimentally induced CLA.

MATERIALS AND METHODS

Bacterial strains and culture. C. pseudotuberculosis WA1030 was grown at 37°C in brain heart infusion broth (Difco) or on the same medium solidified with 1.5% Bacto Agar (Difco). A number of field isolates of C. pseudotuberculosis designated A01 to A05 were also used in this study.

Sheep. All animals were from a CLA-free flock of Romney-Marsh sheep bred at the CSL Field-station, Woodend, Victoria, Australia. Adult sheep, used for immune response studies, were housed inside and fed a diet of chaff and pellets. Lambs, used for the vaccine trial, remained in paddocks.

Preparation of ASC probes. To study the local immune response to infectious challenge with C. pseudotuberculosis, ASC probes were prepared by culture of ASC following infection. Naïve adult sheep that had not previously been vaccinated against CLA or infected with this bacterium were used. Sheep were infected by subcutaneous injection of 1 ml of dilute suspension of caseous material obtained from a CLA lesion; the suspension was prepared by homogenizing approximately 0.1 g of excudate with 1 ml of phosphate-buffered saline and contained 6.8 x 10⁶ CFU of C. pseudotuberculosis per ml. The subcutaneous injection was given on the outer aspect of the right hock, resulting in a local infection, detected as a swelling within 7 days. Following infection, sequential samples of blood were taken and sheep were killed by intravenous injection of 30 ml of sodium pentobarbitone (Lethabar) either 7 or 14 days after infection. The popliteal lymph node was removed by previous tracer studies with Evans blue dye to stain the area of infection. The left and right popliteal lymph nodes were dissected out, and mononuclear leukocyte suspensions were prepared on Ficoll-Isopaque gradients. The mononuclear cells, containing ASC, were cultured at 2 x 10⁷ cells per ml in Difco’s modified Eagle medium (Difco) containing 10% (vol/vol) heat-inactivated fetal calf serum, 2 x 10⁻⁵ M 2-mercaptoethanol, and the antibiotics penicillin (100 U/ml) and streptomycin (100 µg/ml). Mitogens were added to ASC cultures at the following concentrations: pokeweed mitogen (Gibco), 25 µg/ml, final concentration; lipopolysaccharide (LPS; Sigma), 50 µg/ml, final concentration; and dextran sulfate (D.SO₄; BDH) 20 µg/ml, final concentration. After 7 days of culture at 37°C in a 5% CO₂ incubator, cells were removed by centrifugation (1,000 x g, 10 min), and the supernatants were retained. These supernatants obtained by culture of ASC are referred to as ASC probes.

Antigen identification. ASC probes were used to screen whole-cell antigens of C. pseudotuberculosis by immunoblots. Washed whole cells (10 µl of cells per lane at an optical density at 600 nm [OD₆₀₀] of 1.0) were solubilized with loading buffer, run on 12.5% (wt/vol) sodium dodecyl sulfate (SDS)-polyacrylamide gels (Mini-Gels; Bio-Rad) under reducing conditions using a discontinuous buffer system, and then electroblotted onto nitrocellulose (BA35; Schleicher & Schuell). After blocking overnight in Tris-buffered saline (pH 7.4) with 0.05% (vol/vol) Tween 20 (TBS), blots were incubated with either serum (diluted 1/100 in TBS) or ASC probe (undiluted) for 1 h at room temperature. After four washes in TBS, bound sheep antibodies were detected by incubation with an anti-sheep horseradish peroxidase conjugate (Dako) diluted 1/600 in TBS followed by washing and incubation in diaminobenzidine substrate (Sigma; 2 mg/ml) and H₂O₂ (20 mM) in citrate buffer, pH 5.5.

Antigen purification. The 40-kDa antigen identified by ASC probes was prepared from 50 liters of culture supernatant that had been concentrated by pressure dialysis. Ammonium sulfate was added to 50% saturation, and precipitated proteins were removed by centrifugation. The supernatant was applied to a phenyl-Sepharose hydrophobic chromatography column (Pharmacia) and eluted with a decreasing (NH₄)₂SO₄ gradient. ASC probes were used to monitor fractionation of the 40-kDa antigen preparation.

Vaccination trial. Sixty-four wether lambs, approximately 6 months old, were randomly allocated to one of three groups and were identified by ear tags. Sheep in group 1 received 100 µg of 40-kDa antigen preparation in aluminum hydroxide, group 2 animals received 5 µg of 40-kDa antigen preparation per dose given in aluminum hydroxide, and group 3 sheep received a placebo injection of aluminum hydroxide alone. Each animal was given two subcutaneous injections 4 weeks apart. Three weeks following the second vaccination, sheep were bled and challenged by subcutaneous injection of a washed suspension of 2 x 10⁷ in vitro-grown C. pseudotuberculosis cells in the lower right back leg. Three months after infectious challenge, the sheep were slaughtered at a commercial abattoir. As ears and tags were removed before inspection, sheep were identified by consecutive numbering of the gambrels or hangers, and these numbers were coded to ear tag numbers for later analysis. Carcasses were examined by Department of Primary Industry inspectors. Lungs were removed and placed in plastic bags for subsequent scoring of lesions. Lesions were detected by palpation, and any enlarged lymph node or swelling in tissue was examined by serial incisions. After all tissues had been examined and results had been recorded, gambrel numbers were decoded and group lesion scores were collated.

Assay of serum antibodies. Antibody responses in sera were assayed by enzyme-linked immunosorbent assay (ELISA). Whole cells diluted to an OD₆₀₀ of 0.01 or culture supernatant of 40-kDa antigen preparation diluted to approximately 5 µg of protein per ml in bicarbonate buffer, pH 9.6, were dispensed in 100-µl aliquots into 96-well microtiter plates (Nunc Immunoplate). After coating overnight at 4°C, wells were washed and blocked with TBS for 1 h. Dilutions of sera were incubated for 1 h at 37°C, wells were washed, and 100-µl aliquots of anti-sheep peroxidase conjugate (Dako) diluted 1/5,000 in TBS were added. Conjugate was incubated for 1 h, wells were washed with TBS, and bound conjugate was detected with 100-µl aliquots of tetramethylbenzidine (100 µg/ml; Aldrich) substrate with 20 mM H₂O₂. ELISA reactions were stopped with 50 µl of 2 M H₂SO₄.

Statistical methods. Results of the vaccination trial were analyzed by standard statistical methods (10).

RESULTS

Antigen identification. Sequential serum samples taken from naïve (CLA-free) sheep during the course of a primary infection were screened by immunoblots against whole-cell antigens. Serum antibody responses are shown in Fig. 1a. Little antibody response was detected during the first 4 to 5 days (Fig. 1a, panels i and ii). A band at approximately 28 kDa observed with preinfection sera from naïve sheep increased in intensity with sera from day 4 of infection onwards. This
antigen was identified as a major cell protein of 28 kDa on SDS-polyacrylamide gel electrophoresis (PAGE) of whole cells (Fig. 2a, lane 1) that gave a background reaction on immunoblots. Other minor bands did not increase in intensity from days 2 to 7 of infection (Fig. 1a, panel i). Infections of C. pseudotuberculosis that were allowed to proceed for 14 days stimulated antibodies to a wide range of antigens, the immunoblot patterns of sera becoming more complex from day 7 onwards, as the infection progressed.

ASC probes prepared from lymph nodes taken from sheep killed at day 7 of infection showed little reactivity on immunoblots (Fig. 1b, panel i). The background band at 28 kDa observed on immunoblots with preinfection sera was also seen with ASC probes prepared from lymph nodes draining infected sites of sheep killed at day 7 (Fig. 1b, panel i) and from lymph nodes draining uninfected sites (Fig. 1b, panel ii). ASC probes from sheep killed 14 days after a primary infection showed strong and restricted reactivity on immunoblots to whole-cell antigens (Fig. 1b), especially compared with serum antibody. Probes from ASC draining the infected site were strongly reactive, while those from the contralateral uninfected site showed background reactivity to the 28-kDa protein (Fig. 1b).

The spectrum of reactivity of ASC probes at day 14 to antigens of C. pseudotuberculosis showed some variation between sheep, with a number of bands highlighted by some sheep (Fig. 1b, panels iii and iv). However, a 40-kDa antigen was consistently and strongly recognized on immunoblots and in two sheep was the major antigen recognized (Fig. 1b, panels ii and iii). Analysis of immunoblots of sera showed that infected sheep also had circulating antibody to the 40-kDa antigen, and sequential serum samples showed this was one of the earliest antigens recognized during the course of infection (Fig. 1a, panel ii). ASC probes also strongly recognized the 40-kDa antigen in concentrated culture supernatant (Fig. 2b), with a minor band at 38 kDa also visible, which we believe to be a smaller form of the same antigen. Polyclonal sera raised to the 40-kDa antigen preparation also recognized the 38-kDa band, seen in Fig. 2c as the lower band of a doublet.

ELISA of antibodies in serum taken at day 14 and in ASC probes from the same sheep to whole-cell and supernatant antigens of C. pseudotuberculosis confirmed the results obtained from the immunoblots (Tables 1 and 2). Antibody
responses developed in sera from day 7 onwards. Antibody reactivity of the probe from the challenged site reacted strongly with antigens at day 14, but the ASC probe from 7-day infections was negative, as were probes prepared from lymph nodes draining uninfected sites. Other experiments have shown that inclusion of mitogens in cultures leads to a higher level of antibody, as measured by ELISA, but does not affect the limited spectrum of antigens recognized by ASC probes on immunoblots.

Antigen preparation. The 40-kDa antigen was fractionated from 50 liters of culture supernatant by hydrophobic chromatography with a decreasing gradient of 50 to 0% saturated ammonium sulfate. The 40-kDa antigen eluted between 25 and 15% saturated (NH₄)₂SO₄ and was approximately 50% pure as judged by scanning densitometry of Coomassie-stained SDS-PAGE gels (Fig. 2a, lane 3); this is defined as the 40-kDa antigen preparation. The major contaminating moieties were peptides of less than 20 kDa derived from the culture medium. The large scale precluded some techniques, such as gel chromatography, for further purification. Silver staining highlighted a red-to-rust brown diffuse band at the top of the gel. The 31-kDa PLD toxin was the major protein present in bacterial culture supernatants (Fig. 2a, lane 2) but could not be detected either on stained gels of the 40-kDa antigen preparation or on immunoblots of sera from sheep immunized with the 40-kDa antigen preparation; such blots showed that only the 40-kDa antigen was immunogenic (Fig. 2c). The contaminating bands of <20 kDa visible in Fig. 2a, lane 3, were not immunogenic when reacted with ASC probes (Fig. 2b) or with the sheep sera (Fig. 2c). Six field isolates of C. pseudotuberculosis were shown by immunoblotting experiments using ASC probes to also contain the 40-kDa antigen (not shown).

**Immune response to vaccination.** Antibody titers of vaccinated sheep were measured by ELISA prior to infectious challenge. Following primary and secondary vaccinations, there was no difference in the group mean titers to the 40-kDa antigen of sheep vaccinated with 5 μg of antigen (mean OD₄₅₀ ± standard deviation = 0.318 ± 0.166) compared with those of sheep vaccinated with 100 μg of antigen (0.294 ± 0.177). Unvaccinated control sheep had significantly lower titers (OD₄₅₀ ± standard deviation = 0.075 ± 0.122). Immunoblots of sera from vaccinated sheep showed a strong response only to the 40-kDa antigen (Fig. 2c).

**Lesion scores.** Infectious challenge with C. pseudotuberculosis in the right hock 3 months prior to slaughter resulted in a satisfactory level of infection, with 55% of control animals developing lesions (Table 3). Vaccination with 100-μg doses of the 40-kDa antigen preparation had a major effect on development of disease and significantly reduced the infection rate from 55 to 5% (z test value = 3.58, P < 0.002). Vaccination with 5-μg doses of antigen resulted in a small and insignificant reduction in the infection rate to 41% (z test value = 1.25, P < 0.20).

Lung lesion scores provided the best measure of protection achieved by vaccination with the 40-kDa antigen preparation. More control sheep developed lung lesions than developed lesions in lymph nodes, and those infected often had multiple lesions in the lung (Table 3). Of the nine control sheep with lung lesions, five did not have lesions in popliteal or other lymph nodes.

Vaccination with 100-μg doses of the 40-kDa antigen preparation gave a significant 98% reduction in the incidence of lung lesions (unpaired t test, t = 2.046, df = 41, P = 0.025; Table 3). The 5-μg dose showed a 79% reduction (t = 1.932, df = 26, P = 0.05). Vaccination also tended to reduce the lesion size, with control group lesions having an average diameter of 67 mm and lesions in the 5- and 100-μg dose groups having average diameters of 37 and 26 mm, respectively. These data show that in addition to preventing actual infection as reflected in the number of lung lesions, vaccination with the 40-kDa antigen preparation also limited the development of established foci of infection.

The right popliteal lymph node drains the site of infectious challenge; however, only 5 of the 22 right popliteal lymph nodes from control sheep showed lesion development. Vaccination of sheep with the 40-kDa antigen preparation at 5- and 100-μg dose levels prevented infection in the right popliteal lymph node (Table 3). The only other lymph nodes with lesion development were the prescapular nodes. Three control sheep and one sheep in the 5-μg dose group had lesions in the prescapular lymph nodes. No sheep in the 100-μg dose group had detectable lesions in peripheral or visceral lymph nodes.

### Table 1. Titers in serum

<table>
<thead>
<tr>
<th>Day postinfection</th>
<th>ELISA OD₄₅₀</th>
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<tbody>
<tr>
<td></td>
<td>Whole cells</td>
</tr>
<tr>
<td>0</td>
<td>0.580</td>
</tr>
<tr>
<td>2</td>
<td>0.545</td>
</tr>
<tr>
<td>4</td>
<td>0.544</td>
</tr>
<tr>
<td>7</td>
<td>0.620</td>
</tr>
<tr>
<td>11</td>
<td>—</td>
</tr>
<tr>
<td>14</td>
<td>—</td>
</tr>
</tbody>
</table>

* All sera were assayed at a 1/100 dilution.

### Table 2. Titers in ASC probes

<table>
<thead>
<tr>
<th>Origin of probe</th>
<th>Mitogen</th>
<th>ELISA OD₄₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7-Day infection</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Whole cells</td>
</tr>
<tr>
<td>Challenged site</td>
<td>PWM</td>
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</tr>
<tr>
<td></td>
<td>LPS-D.SO₄</td>
<td>0.151</td>
</tr>
<tr>
<td>Unchallenged site</td>
<td>PWM</td>
<td>0.149</td>
</tr>
<tr>
<td></td>
<td>LPS-D.SO₄</td>
<td>0.225</td>
</tr>
</tbody>
</table>

* ASC probes were assayed at a 1/2 dilution.

* PWM, pokeweed mitogen.

### Table 3. Field trial lesion scores of sheep 3 months after infectious challenge with C. pseudotuberculosis

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of infected sheep/no. in group</th>
<th>No. of sheep with popliteal lymph node lesions</th>
<th>Mean no. of lung lesions ± SD (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40-kDa antigen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 μg</td>
<td>2/21*</td>
<td>0</td>
<td>0.1 ± 0.5 (1-2)*</td>
</tr>
<tr>
<td>5 μg</td>
<td>9/22</td>
<td>0</td>
<td>1.4 ± 2.6 (1-12)*</td>
</tr>
<tr>
<td>Unvaccinated controls</td>
<td>12/22</td>
<td>5</td>
<td>6.7 ± 12.6 (1-46)</td>
</tr>
</tbody>
</table>

* Significantly different from control group; see text for details.
DISCUSSION

Antibody probes produced by ASC taken from the lymph nodes draining the site of infection with *C. pseudotuberculosis* showed restricted antigenic reactivity on immunoblots, particularly compared with the diverse spectrum of antigens identified by antibodies from serum present in the sheep at the time of sampling of lymph nodes. This restricted response identified a novel 40-kDa antigen from *C. pseudotuberculosis*. The kinetics of the serum immune response of naive sheep also highlighted the 40-kDa antigen as one of the predominant antigens recognized (Fig. 1a) at day 7 postinfection. By day 14, when ASC from lymph nodes were obtained, the serum response was no longer obvious against other antigens on immunoblots. Taken together, the restricted response of ASC probes and the more heterogeneous response in serum show that the greater part of serum antibody to an infectious challenge is not being synthesized in the draining lymph nodes. The pool of antibodies in serum specific for antigens other than the 40-kDa antigen are presumably being synthesized in other lymphoid tissues, like the spleen.

Vaccination of sheep with 100 μg of the 40-kDa antigen preparation gave up to 82% reduction in the proportion of infected sheep. This level of protection compares favorably with the levels of protection achieved with currently available commercial vaccines (5, 7). The lower dose level of 5 μg of 40-kDa antigen preparation did not provide a significant level of protection in terms of infected sheep but did significantly reduce the incidence of lung lesions. This lower level of protection was not reflected in lower group mean titers of antibody, with group means of the 5- and 100-μg dose groups not being significantly different. In addition, there was no discernible relationship between lung lesion scores and antibody titers. These data strongly implicate cellular immunity as the primary mechanism of immune rejection of infection.

Immunoblot analysis of sera from sheep vaccinated with the 40-kDa antigen preparation showed a strong antibody response restricted to the 40-kDa protein (Fig. 2c). The lower-molecular-mass components of approximately 20 and less than 15 kDa in the 40-kDa antigen preparation were not immunogenic. These were assumed to be derived from medium components of bovine origin. There was no response detected to the 31-kDa PLD toxin. We have not analyzed the cellular immune response to vaccination. The significant protection achieved by vaccination suggests that the 40-kDa antigen plays a major role in protection against CLA. To date, the only other recognized protective antigen has been PLD, which is also a major determinant of virulence (5, 13, 14, 17).

Currently, vaccines against CLA are based on culture supernatants containing inactivated PLD toxin (5). Protection is attributed to responses to the PLD toxoid, and vaccination with purified inactivated PLD has achieved approximately 79% reduction in infection, similar to the 75 to 80% levels of protection achieved with the crude complex commercial vaccines (5–7). In other studies, we have found that antibody responses of sheep vaccinated with one particular commercially available vaccine, Glavac (CSL Ltd), showed low and inconsistent titers to the 40-kDa antigen. Variability in cellular or humoral responses to the 40-kDa antigen may provide an immunological basis for the intermediate levels of protection achieved with commercial and experimental vaccines (1, 2, 5, 7). As discussed previously, cell-mediated immunity may play a role in protection against intracellular infection with *C. pseudotuberculosis*. The absence of a direct correlation between antibody titers to the 40-kDa antigen and disease status indicates either that antibody to the 40-kDa antigen is involved in protection through more indirect mechanisms, for example, antibody-dependent cell-mediated cytotoxicity, or that antibody is not involved in protection. Cytotoxic T-cell responses to the 40-kDa antigen may mediate protection against CLA, as has been shown in other intracellular bacterial infections. There is also the possibility that T-cell responses to antigens that do not elicit an antibody response mediate protection. Further studies are required to elucidate the contributions of humoral and cellular immunity in protection against ovine CLA.

The function and location of the 40-kDa antigen are not yet known. The antigen is present at significant levels in culture supernatant and in detergent extracts of whole cells and is probably noncovalently attached to the cell or is actively secreted. The higher reactivity in ELISAs of ASC probes specific for the 40-kDa antigen with supernatant antigens than with cellular antigens (Table 2) supports the concept that the 40-kDa antigen is a secreted protein. Studies are continuing to clone the gene for and express the 40-kDa antigen and determine its role in immunity and pathogenesis. The identification of a major protective antigen of *C. pseudotuberculosis* other than PLD should contribute to the development of an efficacious defined vaccine against CLA.

Studies of serum antibody responses following natural infection and vaccination have highlighted a wide range of antigens from *C. pseudotuberculosis* (8, 9). These previous studies showed a number of antigens in the 40-kDa region on immunoblots, but the strong response to a very wide range of antigens prevented the selection of any particular antigen as being important in immunity. Identification of protective antigens by immunoscreening using serum antibodies has yielded important candidate vaccine antigens, for example, paramyosin of *Schistosoma mansoni* (18). In previous studies of CLA (9) and in mycobacterial infections, which like CLA are caused by facultative intracellular pathogens, effective vaccine antigens have not yet been identified. We have shown here that ASC probes generated by ASC taken from lymph nodes draining the site of a challenge infection with *C. pseudotuberculosis* identified a highly protective antigen. This study demonstrates that generation of ASC probes from a site of infection provides a rational approach to the identification of candidate vaccine antigens which may have application to a wide range of diseases of both humans and animals.

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