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Cloning, Expression and Purification of Truncated *Chlamydia Trachomatis* Outer Membrane Protein 2 (Omp2) and its Application in an ELISA Assay

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ABSTRACT

Background: Although a simple and direct method does not exist for the detection of chlamydial infections, there are situations in which reliable serological tests, with sensitivity related to a specific antigen, could be helpful. **Objective**: The aim of this study was to clone the first 1100 bp of the *C. trachomatis* outer membrane protein 2 (*omp2*) gene in order to prepare a recombinant protein for use in an ELISA system designed to recognize the anti- *C. trachomatis* antibody in patient sera. **Methods:** The PCR product of the chlamydial *omp2* gene was cloned in pBluescript and its first 1100 bp was subcloned in the pQE-30 expression vector and induced by IPTG. The recombinant protein was purified by affinity chromatography and its purity was confirmed by SDS-PAGE, gel diffusion and western blot analyses. The purified protein was coated onto a polystyrene microplate and tested by ELISA using patient serum. **Results**: We have cloned, over-expressed and purified biologically functional recombinant truncated Omp2 from *C. trachomatis* for use, as a species-specific recognition antigen, in an ELISA system. In this study we determined a cut-off value of 0.345 for this ELISA system using 55 negative sera and measured six positive sera at dilutions of 1:20-1:2560. **Conclusion:** As a species-specific recognition antigen, the over-expressed and purified recombinant truncated Omp2 from *C. trachomatis* performed well in an ELISA system.

Keywords: *Chlamydia trachomatis*, Omp2, Recombinant protein, ELISA

INTRODUCTION

C. trachomatis genital infections are among the most important causes of sexually transmitted diseases (STDs). They often manifest themselves as prostatitis and epididymitis in men, and as cervicitis, endometriosis, vaginitis and urogenital tract infectionsin women.

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Furthermore, urogenital infections with this bacterium are frequently unnoticed and long-term infections can result in infertility (1).

Several antigens, including Omp2, have been used in ELISA systems for the detection of *C. trachomatis* infection.

The outer membrane protein 2 (Omp2) of *C. trachomatis* was described first by Allen et al. in 1989 (2) and then characterized by Coles et al. (3). Allen reported that the 60-kDa cysteine-rich Omp2 from *C. trachomatis* participates in the disulfide-mediated outer-membrane organization unique to this organism. They proposed this protein to be the primary focus of the host immune response (4).

Omp2 is an immunodominant antigen giving rise to antibody responses which have been detected by ELISA in humans infected with *C. trachomatis* serovars A-K. The sensitivity of this assay was high, but varied depending on the gold standard applied. Bas et al. have evaluated various immunoassays using recombinant antigens or synthetic peptides for serodiagnosis of *C. trachomatis* infection (4). They found ELISA systems using particular segments of Omp2 protein available to them at that time to be sensitive but not specific.

Patient antibody response has been used in the diagnosis of chlamydial infections in addition to the culture of the organism from patient specimens. This procedure is difficult and can be performed only in specialized laboratories through detection of the chlamydial genome by PCR or detection of the chlamydial antigen by enzyme immunoassay.

Although serological methods cannot replace procedures aimed at the direct detection of *C. trachomatis*, there are cases in which reliable serological tests can be helpful. Such tests include diagnosis of *C. trachomatis* infections when a test for direct detection of bacteria is negative or difficult to perform, as in the case of upper genital tract infections (5).

An ideal antigen such as the major outer membrane protein would be recognized by all patients infected by a particular species such as *C. trachomatis*. However, it would be desirable to use an antigen that is not recognized as a part of the immune response in other *Chlamydia* species (4, 6, 7). Assays for the cysteine-rich protein Omp2 have been found to be more sensitive and specific than those for other antigens, like Momp and Hsp60 (8).

In this study, we performed a search for a segment of the Omp2 sequence unique to *C. trachomatis,* cloned and expressed this segment and used it in an ELISA system to detect anti-chlamydial antibodies in patient sera.

MATERIALS AND METHODS

Sampling. Peripheral blood samples were collected from patients found positive for *C. trachomatis* by PCR method in a study in which asymptomatic women were randomly selected from patients attending the gynecology out-patient clinic of Hazraat-e Rasool Hospital in Tehran. The PCR product of all patient samples was confirmed by restriction analysis (9) and reconfirmed by Medoc, a specific diagnostic kit based on a synthetic peptide of an immunodominant region of Momp. Serum was separated by centrifugation and stored at -20ºC.

Primer Design. Specific primers were designed based on the gene sequence of the 1644-bp *Chlamydia omp2* gene (GenBank accession no. M23001) containing the start (ATG) and termination codons. *Sac*I and *Bam*HI restriction sites were included at the 5′ end of the forward and reverse primers, respectively, to facilitate the cloning process.

DNA Extraction and PCR Amplification. The cultured *C. trachomatis* was a gift from Dr. Badami, Tehran University of Medical Sciences, The DNA was extracted as previously described (10). The PCR mixture contained 1x PCR buffer, 0.1 mM dNTP, 1.5 mM $MgCl_2$, 0.2 μg of DNA, 20 pmol each of the forward and reverse primers, and 1.5 units of *Taq* DNA polymerase (CinnaGen, Iran). The final volume of the reaction mixture was adjusted to 50 μl with distilled water. The PCR cycling program was: denaturation at 94 °C for 30 sec, annealing at 52 °C for 30 sec and extension at 72 °C for 45 sec. These processes were repeated for 30 cycles. The reaction mixture was kept at 94 ^{o}C and 72 ^{o}C, each for 5 min, before and after PCR cycling, respectively (11).

Electrophoresis. The PCR product was electrophoresed in 1% agarose gel. The gel was then stained by ethidium bromide and the DNA band visualized under UV light.

Gene Cloning. *Eco*RV blunt-digested pBluescript and the PCR product were electrophoresed in a 1% low melting point (LMP) agarose gel (12). The DNA bands were then cut out under long-wave UV light and recovered with a DNA purification kit (Fermentas, Lithuania, LT-2028 Vilnius, cat. no. k0513). The recovered *Eco*RV blunt-digested pBluescript was 3′-tailed using dTTP by terminal deoxynucleotidyl transferase (13, 14). The PCR product was ligated to pBluescript (15) by the T/A cloning method, transformed into *Escherichia coli* XLI-blue-competent cells (16), and placed onto an LB agar plate containing 50 μg/ml of ampicillin. Colonies were screened by X-gal and IPTG to discriminate between recombinant (white) and non-recombinant (blue) plasmids. We compared *C. trachomatis omp2* nucleotide sequence (GenBank accession number M23001) with *Chlamydia pneumoniae* gene for the cysteine-rich outer membrane protein (GenBank accession number X53511). Using BLAST software, the amino acid sequence of *C. trachomatis* was searched for identity with other proteins. Since *Sac*I has a recognition site at nucleotide position 1100 on the *C. trachomatis omp2* gene, we decided to clone the first 1100-bp fragment for the region with the least cross reactivity with other chlamydial antigens. The recombinant plasmid was digested by *Sac*I, and the 1100 bp DNA band fragment encoding a truncated Omp2 protein was released. The digested plasmid was electrophoresed on a 1% low melting-point agarose gel and the DNA band was cut out under long-wave UV light and recovered with a DNA purification kit (Fermentas, Lithuania, LT-2028 Vilnius, cat. no. k0513). The purified DNA fragment was subcloned into the *Sac*I-digested pQE30 expression vector, which was used to transform *E. coli* XL1-blue-competent cells. The positive colonies containing the expected plasmids (encoding a truncated Omp2 protein with 366 amino acid residues) were then masscultured in LB medium. The recombinant plasmids were extracted (17) and checked by restriction analysis. The recombinant plasmid which had the correct orientation was selected and named pQECTOMP.

Gene Expression. The *E. coli* strain BL21 (DE3) was transformed with plasmid pQECTOMP and selected on LB agar containing 50 μg/ml of ampicillin (Qiagen, Netherlands). The transformed cells were inoculated into a 3-ml tube culture containing modified YT medium (1.2% Bacto-tryptone, 2.4% yeast extract, 0.04% glycerol, 1% M9 salts). M9 salts contained 6.4% $Na_2H_2O_4.7H2O$, 1.5% KH_2PO_4, 0.025% NaCl, and 0.05% NH_4Cl). The cells were allowed to grow overnight at 37 ºC in a shaker at 160 rpm. Then, the cultured bacteria were inoculated into a 50 ml flask and allowed to grow at 37 ºC in a shaker at 200 rpm.

Cultures in the logarithmic phase (OD_{600} = 0.6) were induced for 3 hours with 1 mM IPTG. After induction, the cells were lysed in 5X sample buffer (100 mM Tris–HCl, pH 8, 20% glycerol, 4% SDS, 2% β-mercaptoethanol, 0.2% bromophenol blue) and

analyzed by SDS-PAGE (12% polyacrylamide gel). The gel was stained with Coomassie brilliant blue R-250. The uninduced control culture was analyzed in parallel (Novagen, Netherlands).

Protein Purification. Colonies from LB agar plates were used to prepare preinoculation culture in modified YT medium containing 50 μg/ml of ampicillin. The preinoculum was used to grow 500 ml of cultured cells in modified YT medium with 50 μg/ml of ampicillin at 37 ^{o}C to an OD_{600} of 0.6–0.8 followed by induction with 1 mM IPTG for 3 hours at 37 ^{o}C. After centrifugation at 6500 rpm for 10 minutes, the cell pellet was suspended in 50 ml of equilibration buffer (50 mM Tris, 0.5 M NaCl) containing protease inhibitor cocktail (Sigma, Germany). The cell suspension was sonicated (2 × 30 s) on ice. The cells were harvested by centrifugation at 4000×g for 15 minutes, suspended in 5 ml of ice-cold buffer containing 6 M urea and incubated on ice for 1 hour. The insoluble materials were removed by centrifugation at 12,000×g for 20 minutes. The supernatant was filtered through a membrane (0.45 μm pore size) before binding to the resin. The recombinant protein was bound to the affinity chromatography column packing material by its N-terminal His_6 tag using Ni-NTA His-bind resin. The column was equilibrated with 15 ml of equilibration buffer. The filtered supernatant was dialyzed against PBS to remove urea, and then applied to the column at a flow rate of 15 drops/min. The bound protein was eluted in 500 mM imidazole buffer (50 mM Tris, pH8, 0.5 M NaCl, 0.5 M imidazole). The sample was dialyzed overnight in 500 ml of buffer (50 mM Tris–HCl, pH 8, 50 mM NaCl). This buffer was replaced four times over a period of 24 hours. The concentration of the purified protein was measured by the Bradford method (18). Dot blot and western blot analyses were carried out with the His-tagged monoclonal antibody or the patient serum and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG to estimate the expressed protein in *E. coli* cells collected three hours after induction by IPTG (19). The purified protein was also assayed by gel diffusion with patient serum.

Testing of Recombinant Protein by ELISA. Polystyrene microtiter plates were coated with purified recombinant protein as previously described (20). Briefly, the purified recombinant protein was dialyzed for two hours at 4ºC against carbonate/bicarbonate sodium buffer (0.1 M, pH 9.5) to exchange the chromatography elution buffer with ELISA coating buffer and to remove traces of urea. The absorbance of the protein solution after dialysis was determined at 280 nm (OD=1.6) and diluted up to 20-fold to reach an OD of 0.07, as determined by antigen serial-dilution testing to obtain the optimum amount of antigen to be coated onto the Nunc Maxisorp flat-bottomed 96-well plates. The diluted antigen in carbonate/bicarbonate sodium buffer was coated onto the plates and incubated overnight at 4 ºC. After washing three times with PBS-Tween 20, the 100-fold diluted serum samples of 55 negative volunteers were confirmed as negative by the Viro-Immun Anti-*C. trachomatis*-IgG kit and incubated on the coated antigen for one hour at 37 ºC. After washing and addition of the anti-human IgG conjugate (γ-chain; 1:3000), and incubation for one hour at 37 ºC and then washing six times, the substrate (OPD + H_2O_2) was added, the reaction mixture was incubated for 10 min and then stopped by the addition of 0.5 M H_2SO_4. The OD of the reaction mixture was measured at 492 nm with a 620 nm reference filter using a Tekan ELISA reader (Tekan LTD, Moscow 121105, Russia). The mean value of the negative serum was determined to be 0.138, and the cut-off value was defined as the mean ± 2 SD (0.345).

RESULTS

Figure 1A illustrates the differences between the nucleotide sequence of *C. trachomatis omp2* (accession number M23001) and that encoding the *C. pneumoniae* cysteine-rich outer membrane protein (accession number X53511). The first 917 nucleotides were completely different. Figure 1B compares the 250-amino acid sequence of *C. trachomatis* Omp2 protein with that of *C. pneumoniae*.

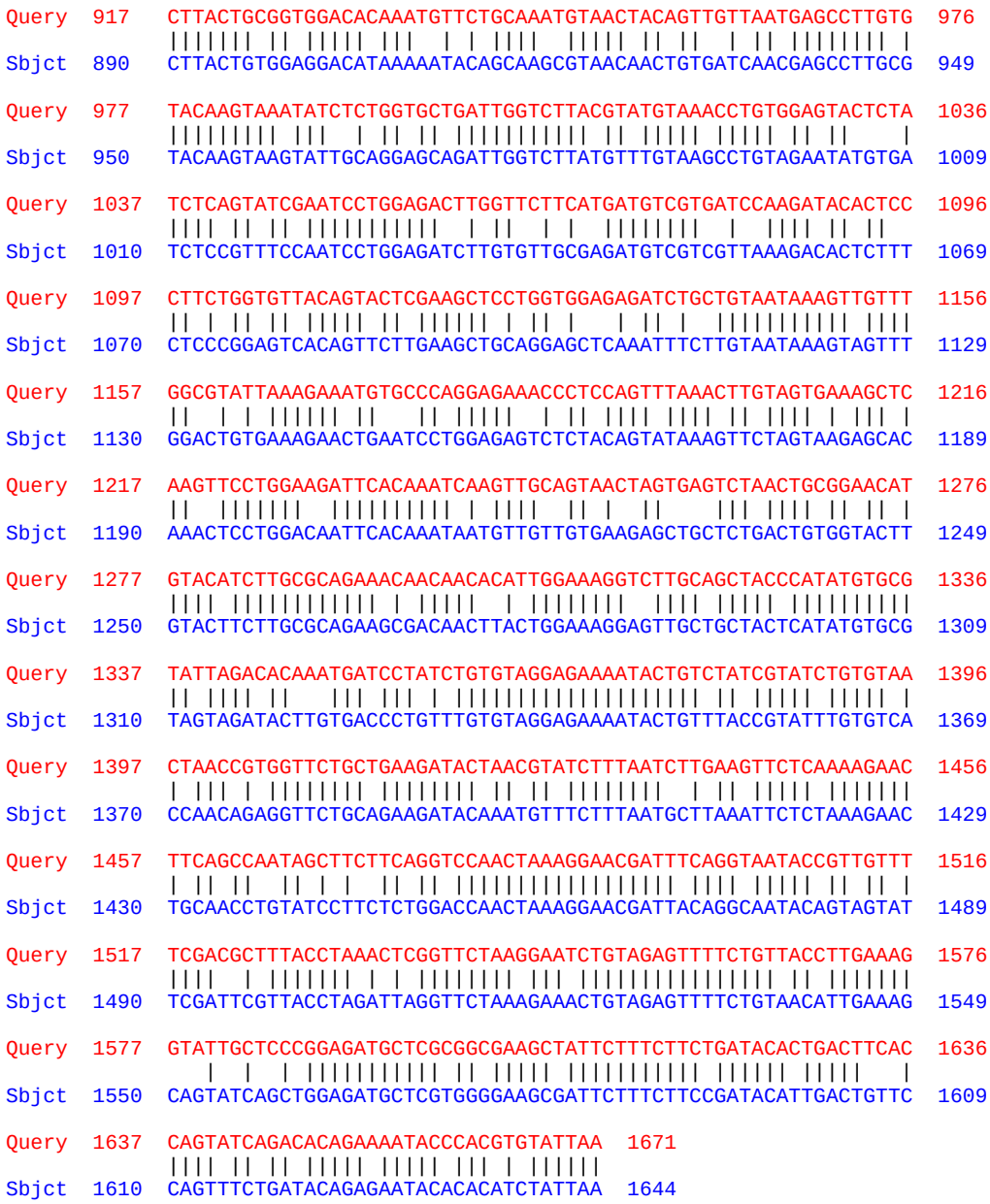

Figure 1A. Comparison of *Chlamydia trachomatis* OMP2 (red) with *C. pneumoniae* gene for cysteine rich outer membrane protein (blue). The colourful version is available at: www.iji.ir

The *omp2* gene was amplified from *C. trachomatis* DNA using specific primers. The identity of the PCR product was confirmed by restriction analysis based on GenBank accession number M23001. Then, the PCR product was cloned into the pQE-30 expression vector and the recombinant plasmid (named as pQECTOMP) was transformed in

BL21 (DE3) *E. coli* and induced using 1 mM IPTG. Samples were taken before and at 3 hour intervals after induction. The identity of the induced protein was confirmed by SDS-PAGE. Figure 2 shows an SDS-polyacrylamide gel loaded with samples taken before and after induction, and the induced protein is observed at approximately 40 kDa.

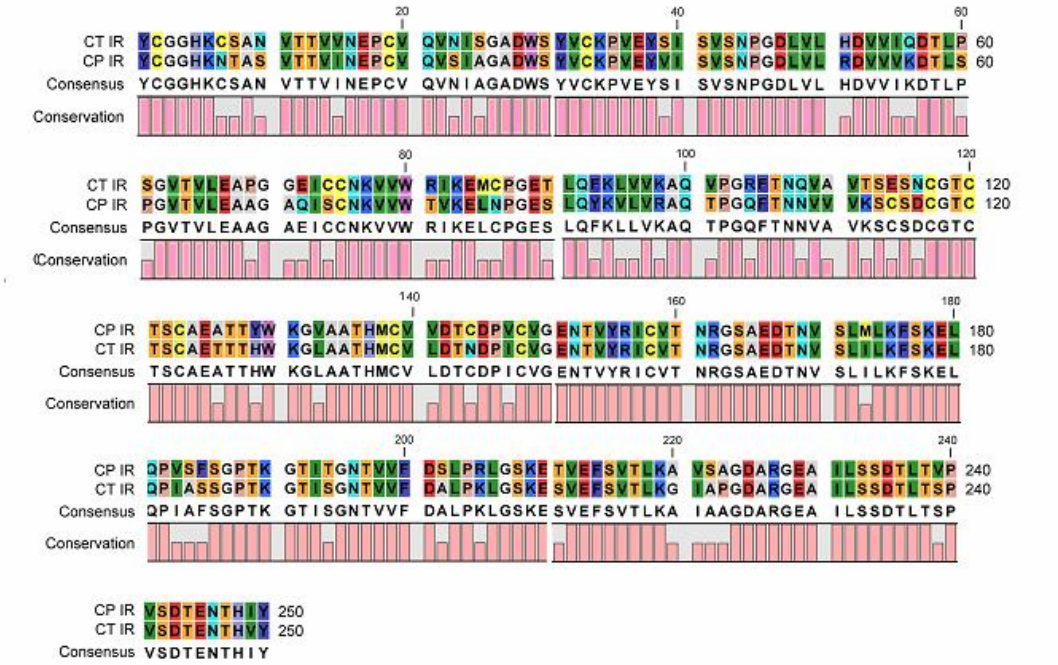

Figure 1B. Comparison of the 250 amino acids of *Chlamydia trachomatis* OMP2 (CT IR) with *C. pneumoniae* OMP2 (CP IR) protein. As illustrated there are differences in the amino acid sequences of the two proteins.

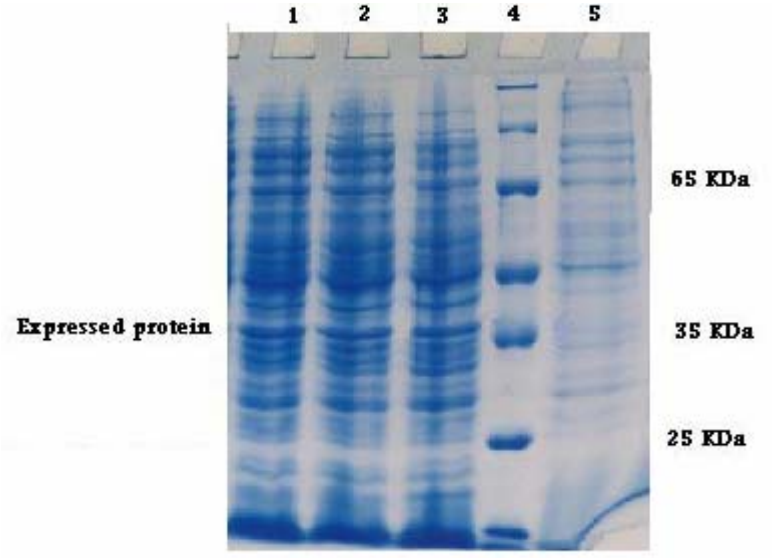

Figure 2. SDS-PAGE of the IPTG induced bacterial cells and the uninduced cells. Lines 1,2 and 3: Bacterial culture 3 h after induction. Line 4: Protein markers, Line 5: Bacterial culture before induction.

The affinity chromatography-purified recombinant *C. trachomatis* Omp2 protein was done in the SDS-PAGE gel stained with Coomassi blue (data not shown). The specificity

of the protein was confirmed by gel diffusion, dot blot and western blot analyses using positive sera (data not shown).
The purified protein was coated onto Nunc Maxisorp flat-bottom 96-well plates and a cut-off value (OD mean of 0.154) was obtained using 55 negative sera; 6 positive sera were then applied by ELISA to give a cut-off value of 0.345 (OD mean of positive sera=1.473). The positive sera produced good results with our truncated recombinant protein. In practice, we measured the positive sera by ELISA at dilutions of 1:20 - 1:2560, and with a cut-off value of 0.345, positive sera at dilutions of up to 1:320 were significantly higher.

DISCUSSION

C. trachomatis, the organism most clearly associated with non-gonococcal urethritis (NGU), is an obligate intracellular parasite that is the cause of as many as 30-50% of NGU cases and lymphogranoloma venerum (LGV), as well as non-genital diseases, such as keratoconjunctivitis and trachoma (one of the leading causes of blindness in the world), which are major health problems in societies with poor hygiene (21, 22).
The most specific method of chlamydial detection is by inoculation of a sample into the yolk sac of a 7 to 8-day-old chicken embryo. Chlamydia can also be grown in a wide range of animal-derived cell lines (McCoy, Hella 229, BAMK and SHK2). However, these procedures are difficult, time-consuming and of little value as routine clinical tests, since early detection and treatment provide the best outcome (23). Thus, immunologic tests that take advantage of recombinant technology to produce specific proteins from the bacteria are preferred.
Goodall et al. produced a recombinant bacterial protein called Omp2, which is rich in cysteine residues, and was useful in detecting the cell-mediated response against *C. trachomatis* in blood and synovial fluid (24).
In using antibody response for the diagnosis of chlamydial infections, systems employing the cysteine-rich protein, Omp2, have been found to be more sensitive and specific than those employing four different antigens, including Momp and Hsp60 (4, 6, 8). The 5′-end sequences of *C. trachomatis* and *C. pneumoniae* DNA are different as demonstrated by Watson et al. (25). We used a pQE-30 expression vector for recombinant protein preparation similar to the works of Portig et al. and Goodall et al. (7,24). However, the present study is the first using this particular truncated Omp2 protein, including the 5′ end, in an ELISA system for the diagnosis of *C. trachomatis* infection. We used positive sera from asymptomatic patients whose specimens were found to be positive in a PCR method to test this ELISA system. All of the sera were also found to be positive by this ELISA method. Thus the truncated protein at the defined concentration is capable of differentiating between positive and negative sera. Although our truncated recombinant protein is satisfactory for the diagnosis of chlamydial infection using a cut-off value of 0.345 and can detect positive sera up to dilutions of 1:320, in order to obtain approval as a diagnostic kit, further investigations are required to determine its sensitivity and specificity. In conclusion, an over-expressed and purified recombinant truncated Omp2 from *C. trachomatis* performed well in an ELISA system as a species-specific recognition antigen.

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