ELISPOT Assay for Chlamydia-specific, Antibody-producing Cells Correlated With Conventional Complement Fixation and Microimmunofluorescence

Harry Daugharty,^{1*} Trudy O. Messmer,¹ and Barry S. Fields¹

¹Respiratory Diseases Laboratory Section, Childhood and Respiratory Diseases Branch, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, National Centers for Disease Control and Prevention, Atlanta, Georgia

Chlamydia antigens cross-reactive with *pneumoniae* (TWAR), *psittaci*, and *trachomatis* strains were used to evaluate the ELISPOT assay for detecting antigen-specific, antibody-secreting cells (ASC). Human blood specimens from healthy and hospitalized persons were randomly collected and tested by coating the nitrocellulose membrane at the base of microtiter wells. Ficoll-separated mononuclear cells from blood specimens collected in EDTA were incubated in the wells with Iscove's growth medium in CO₂ atmosphere at 37°C. An IgG-specific conjugate labeled with biotin was used in an avidin-peroxidase chromogen system for indicating the areas (spots) of immunologically committed lymphocytes. Positive specimens had median levels of ASC above 8 per 10⁶ cells (range 15–23 ASC/10⁶ cells). Evidence that the ELISPOT is reliable, sensitive, and specific includes the following: (1) immunized animal and clinical human specimens in control experiments were selectively reactive in the presence of antigen, but negative without antigen, (2) serologically characterized

reference sera demonstrated homologous rather than heterologous reactions with the antigens, (3) conventional complement fixation and microimmunofluorescence on serum fractions of clinical specimens correlated well ($P < 0.02$) with ELISPOT results that were both TWAR- and psittaci-positive, and (4) the array of specimens (from healthy donors, community hospitalized, and pulmonary service patients) selected for their increasing likelihood in that order for being positive due to illness was then confirmed and supported by their respectively increasing positivity rates (6, 15, and 25%) for TWAR/psittaci combined. The incidence of positive specimens for either TWAR or psittaci was greatest (23/54, 43%) in specimens from the hospitalized patients and least (8/33, 24%) in specimens from healthy individuals. These findings suggest that ELISPOT detects chlamydial antibody production at the cellular level. ELISPOT positivity thus indicates previous exposure and would favor earlier detection. J. Clin. Lab. Anal. 11:45–52. © 1997 Wiley-Liss, Inc.*

Key words: ELISPOT; chlamydia; antibody-producing cells

INTRODUCTION

Chlamydial infections have been numerous and varied. *Chlamydia pneumoniae* can cause an average of 10% of cases of community-acquired pneumonia, and the vast majority of adults have serologic evidence of past chlamydial infection (1). Serologic tests for *C. psittaci* serology can be positive in 16–23% of individuals (depending on age group) with respiratory tract infections (2). *C. trachomatis* has been shown to induce proliferation and differentiation of human peripheral B lymphocytes. These studies have demonstrated that up to 50% of peripheral lymphocytes bind the chlamydial antigen (3). We presume this binding is from circulating immune complexes of *Chlamydia* binding to Fc receptors (4), or from antigen-specific, antibody-secreting cells (ASC) binding the

chlamydial antigen. Other studies have already demonstrated *Chlamydia*-specific, Ig-secreting cells for ocular infections (5).

It is hypothesized that binding of respiratory *Chlamydia* organisms to human pulmonary lymphocytes and perhaps even to peripheral circulating lymphocytes with antibody as mediator occurs during the course of infection. Such binding may influence the persistence of the organism and the outcome of the disease. This could be

*Correspondence to: Harry Daugharty, Respiratory Diseases Lab Section, Childhood and Respiratory Diseases Branch, Division of Bacterial and Mycotic Diseases, NCID, National Centers for Disease Control and Prevention, Atlanta, GA 30333.

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assessed by the insoluble enzyme-linked immunosorbent assay (ELISA) technique, called ELISPOT (5,6), for detecting ASC with their low levels of nascent *Chlamydia*-specific Ig. Similar assays of ASC (7), e.g., antigen-antibody complexing (8) or direct antigen detection (9), have been used for prognosticating (8) and monitoring (7,9) human immunodeficiency virus (HIV) infections.

The ELISPOT assay has been applied to studies of the immune response (10). Further ELISPOT applications have been successful in species differentiation (11,12). Previously, differentiating *C. pneumoniae* (TWAR) from *C. psittaci* infections by conventional complement fixation (CF) (13) and microimmunofluorescence (MIF) has met with limited success (2,14). More refined methods of ELISPOT have been developed (15,16) that might be applied for earlier detection of chlamydial antibody production at the molecular level for the single cell. We compared the ELISPOT technique for detection of antibody production by specimens from various sources with conventional CF and MIF serologic methods. As with any test, there are limitations with ELISPOT with additional standardization, and clinical correlations that need to be substantiated. However, the test is useful in assessing immune status as an index of prior exposure and possibly in an earlier time frame.

MATERIALS AND METHODS

Specimen Donor Populations

Human subjects (N=87) were selected as donors from three separate populations. Thirty-three healthy donors were selected as a control group from HIV-negative volunteers from the blood services of the Centers for Disease Control and Prevention (CDC). The other two groups of specimens were obtained from hospital patients: one group was a random selection of 46 patients having blood specimens (with EDTA) drawn in a community hospital setting. The other group of eight was a select number of patients with respiratory disorders from the pulmonary service of a Veterans Administration (VA) hospital. Blood specimens consisting of 3–5 ml were collected in EDTA to prevent clotting. Tubes were kept at 4°C until used in the laboratory for cell separation.

Animal studies were conducted on Balb/c mice that had been immunized with chlamydial antigen consisting of elementary bodies from tissue culture growth of the organism. Mice were immunized initially with 50 micrograms of the antigen protein, then boosted 10 days later. After 3 days, the animals were sacrificed and their spleens removed. Spleen cells were separated by mincing and aspirating tissues in Iscove's cell culture medium. The cell suspensions were centrifuged at $\sim 100 \times g$ for 5 minutes to remove tissue debris. The supernatant cells were then used in the ELISPOT assay.

Antigen Preparations

The following test antigens were used in the ELISPOT assay: tetanus toxoid, *Candida albicans*, and the TWAR (TW183) and 6BC strains of *Chlamydia pneumoniae* and *psittaci*, respectively. The tetanus toxoid and *Candida* antigens were obtained from Cutter Laboratories (Berkeley, CA). The tetanus antigen, lot # LP501P was at 875 Lf/ml. The *Candida* antigen, lot # D4656M was a 10% solution (wt/vol) in 50% glycerin as preservative. These antigens were used at 1:250 dilutions for coating microtiter plates in the ELISPOT assay.

Chlamydial antigens (elementary bodies) were prepared by the following procedure (17): inocula of individual strains were diluted in Hepes-sucrose-calcium buffer for inoculating monolayers of HEP2 cells. Inoculum was centrifuged onto the monolayers at $3,000 \times g$. Supernatant was replaced with either Minimal Eagles Essential or Iscove's medium with 10% fetal calf serum, vancomycin, and cyclohexamide (0.14%). After 2–3 days growth at 36°C in CO₂ atmosphere, infected cells were harvested, sonicated 10–15 seconds, then centrifuged at $100 \times g$ to remove cell debris. Supernatant was further purified on a renografin cushion by centrifugation at 17 K rpm, 4°C, for 30 minutes. The pellet was washed twice by centrifugation and finally resuspended in 1.5 M phosphate-buffered saline (PBS), pH 7.2. Merthiolate was added to a final 0.25% concentration for inactivation of infectivity. This antigen was also used for ELISPOT and MIF assays.

Complement Fixation Assay

The microtiter CF assay was performed using the procedure of the Standardized Diagnostic Complement Fixation Method established by the U.S. Public Health Service, CDC (12). A commercial antigen obtained from Telcolab (New York, NY) was routinely used as the CF antigen. This antigen consisted of an extract from *C. psittaci*, which was standardized against the reference CF antigen of the Biological Products Branch, CDC (Atlanta, GA). Endpoint titers of $>1:32$ were considered as positive for antibodies against the group antigen of *Chlamydia*.

Microimmunofluorescence

Specimens were tested using prepared TWAR antigen and previous assay protocols (13). Serum specimens were tested at 1:256 dilution with endpoint positive results of greater than that considered as positive with single serum specimens and with an anti-IgG conjugate.

ELISPOT Assay

The ELISPOT assay is presented in the flow diagram of Figure 1. Ficoll gradients were performed on 3–5 ml samples of EDTA-collected blood specimens. The ficoll was a Lymphocyte Separation Medium from Organon Teknika Corp. (Durham, NC). Specimens were diluted 1:3

ELISPOT Assay for Antigen-specific Antibody-secreting Cells (ASC)

Day 1 Cell Separation, Plating, and Incubation:

Day 2 Detection and Color Development:

Count and Calculate:

Where, 10 is dilution factor
C is cell count / ml

Fig. 1. Procedural aspects of the ELISPOT assay.

in PBS and overlaid on a 1.8 ml cushion of ficoll in 15 ml plastic centrifuge tubes. Tubes were centrifuged 20 minutes at 2,000 rpm (800 × g). The band of predominantly mononuclear lymphocytes were aspirated and resuspended

in Iscove's medium for washing ×3 by centrifugation 5 minutes at 1,000 rpm (250 × g). Final suspension of cells in Iscove's medium (~1.0 ml) was followed by counting cells (1:100 dilution in PBS) with a standard hemocytom-

eter. Cell count = C, the number ($\times 10^6$) of peripheral blood mononuclear cells (PBMC)/ml.

Microtiter plates (Silent Monitor, BioDyne A, Pall Bio-medical Products Corp., New York), had membrane templates at the bottom of their 96 wells. Wells were coated with antigens in concentrations ranging from 2–5 mg/well in 100 ml aliquots. Plates were incubated with antigen at 37°C for 1 hour. Excess antigen was washed off with 10 additions of PBS to fill the wells. After each addition, excess PBS was discarded. Similarly, uncoated binding sites were saturated with a blocker agent, bovine serum albumin at 2.5% concentration (wt/vol) in 100 ml aliquots.

Following incubation at 37°C for 1 hour and 10 washes with PBS, 100 ml of the prepared cell suspension with known cell concentration was added to the wells. Plates were incubated overnight at 37°C in a 10% CO₂ atmosphere. The next day, the plates were washed 10 times, and 100 ml of IgG-specific biotin-labeled species-specific (goat antihuman or -mouse IgG) conjugate was added at 1:1,000 dilution of the commercially supplied reagent. After 2 hours incubation at 37°C at room temperature followed by 10 washes, 100 ml avidin-peroxidase conjugate also at 1:1,000 dilution was added per well and incubated further at room temperature for 2 hours. Plates were washed again $\times 10$, and substrate 3-aminoethyl-9-carbazole, Sigma Chemical Co., St. Louis, MO) was added at 0.03% (wt/vol) concentration. After the membrane was dried, the color-developed spots were counted per field (one microtiter well) under a dissecting scope with 40 $\times$ magnification.

Data Calculation

With R = number of spots counted/well of the microtiter plate, and C = cell count ($\times 10^6$), and using an aliquot correction factor of 10 used for plating 0.1 ml (instead of 1.0 ml) of the cell suspension, the number of ASC per 10^6 PBMC was determined by the formula:

$$\text{ASC} / 10^6 \text{ PBMC} = R \times 10 / C$$

RESULTS

Chlamydia-specific ASC were demonstrated by the color development of spots, as shown in Figure 2. The cells are from ficoll-separated whole human blood or minced spleen tissue cells from immunized mice. Negative control wells without chlamydial antigen had far fewer spots (less than the $8/10^6$ cells considered the upper limit of negatives) than did wells with antigen.

Further specificity of the TWAR and *psittaci* chlamydial antigens was verified by testing them in the MIF assay with reference sera. The reference sera were from persons with confirmed clinical cases and had known specified homologous titers and low to minimal ($<1:32$) heterologous cross-reactivity either by MIF (for TWAR-reactive sera) or by CF (for the *Mycoplasma*-reactive sera). The TWAR and *psittaci*

antigens used in the ELISPOT tests were both highly reactive ($4+ = >1:128$) with the three separate TWAR-positive reference sera. They were nonreactive with three of four *Mycoplasma*-reactive reference sera (one being weakly reactive at $<1:64$). There was no reactivity of the two *Legionella* reference sera with either TWAR or *psittaci* antigens used in ELISPOT.

With satisfactory specificity from these animal and human models, along with stringent reference sera performing as expected, various specimen populations were tested by the ELISPOT assay. Combined populations of specimens from 87 healthy and hospital donors were tested by ELISPOT with both, *C. pneumoniae* (TWAR) and *C. psittaci*, in addition to two unrelated antigens, tetanus toxoid and *C. albicans*. Seropositive rates were highest (Table 1) in the two chlamydial antigens (23.0% for TWAR and 25.3% for *psittaci*) compared with control antigens. Fewer positive reactions were obtained with the two control antigens, candida demonstrating less positivity than tetanus (7.4 vs. 23.8%). In general, the higher the incidence of positive reactions with each antigen, the wider the range of results for ASC/ 10^6 cells. Positive cells with *psittaci* antigen contained as many as 226 ASC.

ELISPOT assay results for all populations of specimens were compared with conventional CF and MIF assay results. Of the 87 specimens tested, 5.8% were positive by both ELISPOT and by either of the conventional CF or MIF serology methods (Table 2). The ELISPOT assay had only a slightly higher rate of positivity with TWAR and *psittaci* ($17.3\% = 5.8\% + 11.5\%$) for serologically positive specimens than did CF and MIF ($10.4\% = 5.8\% + 4.6\%$ for ELISPOT positive and negative specimens). The one specimen with a titer of 1:64 by CF was also positive by ELISPOT. Only the lower CF-titered specimens (1:32 or less) were negative by ELISPOT. As shown in Table 2, of the nine specimens positive by CF and/or MIF, five (56%) were also positive by ELISPOT with either TWAR or *psittaci* antigens.

In general, individual population groups were increasingly positive for chlamydial antigens in the order of specimens from (1) healthy donors, (2) community hospital, and (3) VA hospital pulmonary service. As indicated in Table 3, the specimens from the community hospital had the highest incidence rate of positivity for the tetanus antigen. With the chlamydial antigens, the community hospital donors were about equally positive for *psittaci* and TWAR, but exceeded the positivity rate with the chlamydial antigens for healthy donors. Specimens from the pulmonary service had the highest rate of positivity with TWAR.

Each of the specimen populations had a higher proportion of specimens positive for either antigen (*psittaci* or TWAR) than for both antigens (Table 4). Yet, in both cases, those positive with either or both chlamydial antigens, the trend held for the order of increasing positivity: healthy donors < community hospital < pulmonary service.

Fig. 2. Spots developed in the ELISPOT assay, magnified 40X at the base of microtiter plate wells for (A) human and (B) mouse antibody-secreting cells (ASC). Antibodies were reactive with Chlamydia antigen used for coat-

ing the upper wells. Cells were nonreactive in the lower wells without antigen coat. Conjugates used for detection were human and mouse Ig-specific, respectively.

DISCUSSION

Several factors in this study supported specificity of the ELISPOT test. These include the use of immunized animal and clinical human models in control experiments with or without antigen (Fig. 2). In both animal and human models,

absence of antigen gave negative results. ASC were detected in experiments where antigen was used (rather than in its absence) in coating the template for cell culture. In addition, highly reliable and characterized reference sera gave expected homologous positive results in the ELISPOT test with minimal to negligible heterologous reactivity. Finally, using se-

TABLE 1. Parameters of Specimens ASC-positive^a for Test Antigens

	Test antigens			
	Tet tox	C. alb	TWAR	Psit
Number tested	80	27	87	87
Number positive	19	2	20	22
Percent positive	23.8	7.4	23.0	25.3
Range ^b	10–70	10–35	9–95	10–226
Median	15.0	23.0	16.5	20.0

^aFor all specimen populations tested (healthy, community hospital, and pulmonary donors).

^bRange (and median) for ASC/10⁶ cells (i.e., positive values exceeding 8 ASC/10⁶ cells).

lect population specimens yielded an increasingly positive rate in donors: healthy population < community hospital < pulmonary service. We have shown elsewhere (18) that positivity for TWAR/psittaci-specific ASC by ELISPOT is often concomitant with positivity with other respiratory antigens (viz., *Mycoplasma*, *Streptococcus*, and *Legionella*).

It is apparent, then, that the ELISPOT assay effectively detects chlamydial antigen-specific ASC. In our population studies with 87 specimens, we found a substantial range of values and prevalences of positive levels of ASC (Table 1). Further, comparison of CF and MIF positivity rates with ELISPOT indicated a slightly higher sensitivity of ELISPOT that correlated with conventional CF and MIF. Only when the ELISPOT-positive specimens for either TWAR- or psittaci-specific ASC alone were excluded did the statistically significant relationships exist (Table 2).

The ELISPOT results were more reactive in considering positive results with either the TWAR or psittaci. It has been noted in other reports (14) that psittaci and TWAR reactivity is more closely related by MIF, but not by CF. The test systems have their peculiarities of detection. The ELISPOT results are more reliable and specific (although maybe less sensitive) when there is agreement just between antibody reactivity with both TWAR and psittaci combined. Tests may measure different parameters of the antibody, as has been noted

TABLE 2. Chlamydia Reactivity^a by ELISPOT and CF or MIF Assays*

ELISPOT results	CF and MIF results	
	Positive	Negative
Positive ^b	5 (5.8%)	10 (11.5%)
Negative	4 (4.6%)	51 (58.6%)

^aNumber of specimens positive or negative with percentage (in parentheses) for the 87 specimens tested by the three assays.

^bIrrespective of CF/MIF results, specimens positive by ELISPOT alone for both TWAR and psittaci were 12.6%, but 35.7% for either TWAR or psittaci. Thus only CF/MIF-negatives were included here that were both TWAR/psittaci-positive.

*Chi-square analysis: $P = >0.01$, <0.02 .

TABLE 3. Population Positivity for Antigen-specific Antibody-secreting Cells (ASC)

Specimen population	Number tested	% Positive for antigen			
		Tet tox	C. alb	TWAR	Psit
Healthy donors	33	11	2	9	21
Community hosp.	46	34	0	26	28
VA hosp - pulmonary service	8	13	–	63	25

in other test systems (19). ELISPOT with the anti-IgG conjugate as used in this study detected late antibody in the immune response that is often missed by CF tests (20), which like most agglutination test systems is predominantly sensitive for early IgM antibody. Precipitation (or nonagglutination) assays predominantly detect late and persisting IgG antibody (13). The one specimen in our study with a high positive CF value was also positive by both CF and ELISPOT. However, specimens with lower CF values were not positive by ELISPOT, but rather were positive by CF alone.

When MIF positivity rates are included with CF, there is a better correlation between the conventional methods combined and ELISPOT. More than half (56%) of the CF/MIF-positive specimens were also positive by ELISPOT.

ELISPOT positivity was low for persons tested with the unrelated “control” tetanus and candida antigens (Tables 1 and 3). There was a positive rate of 7.4% for candida antigens. With the tetanus antigen, there was a higher positive rate of 23% (10) (Table 1). The rate of positives with chlamydial antigens was even higher (Tables 1–4). This higher rate of positivity with chlamydia may be due to the populations studied, i.e., hospital patients vs. normal healthy persons. As would be expected, the test method of ELISPOT being directed at the molecular level of antibody production and at the level of single cell synthesis favors the sensitivity of the method.

The healthy donor population had the lowest incidence of positive results for most all the antigens included in this study (candida, tetanus, and chlamydia) (Table 3). The highest incidence of positive results was in either of the hospital populations (community or VA pulmonary service). There is evidence that the seroprevalence rate for TWAR increases

TABLE 4. Specimen Population Positivity for Either/Both Chlamydial Antigens: TWAR/Psittaci

Specimen population	Number specimens tested	No. (%) percent TWAR/psittaci-positive	
		Either	Both
Healthy donors	33	8 (24%)	2 (6%)
Community hosp.	46	18 (39%)	7 (15%)
VA Hospital, pulmonary service	8	5 (62%)	2 (25%)
Overall	87	31 (36%)	11 (13%)

with age and that seroreactors to chlamydia may approach 60% (20) in some clinics where higher prevalences exist. The high incidence of *Chlamydia* etiology in pulmonary diseases has also been previously referenced (2).

Diagnosis of chlamydial cases as having confirmed TWAR or psittaci etiology always has been problematic because serology by conventional CF or MIF does not clearly delineate species specificity of the cross-reacting chlamydia antibody (1,14,21). The CF and MIF tests overlap to some extent in their chlamydial group antigen reactivity, yet each test maintains some specificity of reaction (14). This overlap appears to occur with ELISPOT as well as with conventional methods (Table 2). Up to 56% of CF/MIF-positives were also ELISPOT positive. However, ELISPOT reactivity for chlamydia in CF-negative specimens may reflect, in addition to greater sensitivity, previous exposure of individuals to multiple antigens of chlamydia with broad specificity of the antibody.

Further, there was a greater difference in positive results for either TWAR or psittaci alone (35.7%) than for both TWAR and psittaci combined (12.6%) (Table 4). This difference may represent recognition of additional species-specific antigens by the newly synthesized antibody from the actively secreting cells in the ELISPOT assay. Species specificity of antibody has been recognized as the necessary factor for strain characterization of both streptococci (22) and chlamydia (23).

It appears possible to develop an ELISPOT assay that would be effective and more useful in distinguishing chlamydial TWAR and psittaci specificity of ASC-produced antibodies. Varying ELISPOT with species-specific antigens (23) is one alternative. Although TWAR and psittaci have group antigens in common, they also appear to react somewhat independently in ELISPOT. This difference may be due to specific immunodominant antigens previously characterized for various species (24).

These factors contribute to peculiarities of test systems. The CF test is less specific and sensitive for chlamydia than the MIF, which in turn is more specific but has problems of sensitivity and specificity (1,14) as well. The ELISPOT assay did correlate better ($P < 0.02$, Table 2) with CF and MIF with the more stringent considerations for ELISPOT being combined TWAR- and psittaci-positive. This restriction may account for a lower sensitivity but enhanced specificity of ELISPOT. Further studies with increased populations will need to be performed to determine a more accurate sensitivity and specificity for this assay.

The ELISPOT assay has obvious specificity, as indicated by human and animal control experiments, reactivities in reference sera, clinical population correlations, and unpublished data on concomitant positive results with other respiratory pathogens. There is the added versatility in ELISPOT for detecting ASC that are producing class-specific Ig (25). In this study we used a conjugate with IgG specificity since IgG antibody persists longer and is more likely being detected

more often. Predominant responses of the IgM type would be indicative of an acute infection, whereas IgG antibody is predominantly detected in cases of prolonged or earlier infections. This differential reactivity of IgG and IgM would be of significance in determining immune status in epidemiologic studies and for confirming etiology.

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