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Cell-Mediated Immune Responses to *Chlamydia trachomatis* in Mothers and Infants[1] (42296)

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Abstract. Cell-mediated immunity to *Chlamydia trachomatis* was studied in pregnant women with chlamydial infection of the cervix, in infants born vaginally to these women, and in infants presenting with chlamydial conjunctivitis. Uninfected pregnant women and their infants were studied as controls. McCoy cell cultures were used to isolate *C. trachomatis* from clinical specimens. Cell-mediated immunity was measured by lymphocyte proliferative responses *in vitro* to stimulation by chlamydial antigens. Chlamydial IgG antibody in serum specimens was detected by a micro-enzyme-linked immunosorbent assay technique. The mean lymphocyte proliferative responses to chlamydial antigens were greater in infected women than in uninfected women both during pregnancy and in the postpartum period. Lymphocyte responsiveness in infected pregnant women, however, was less than in postpartum women. Despite failure to detect chlamydial infection in exposed infants, lymphocyte proliferative responses were greater in umbilical cord blood and later in peripheral blood samples from neonates born to infected mothers than in infants born to uninfected mothers. These responses were also greater in infants with chlamydial conjunctivitis than in infants of uninfected mothers. These data suggest that cellular immune responses to chlamydial antigens are increased in infected mothers and infants and that infants may acquire chlamydial cell-mediated immunity transplacentally.

Chlamydia trachomatis infection of the genital tract is a highly prevalent venereal disease (1). Maternal infection during pregnancy places the infant at risk for acquisition of infection at birth and development of conjunctivitis and pneumonia (2). Immune responses of the host, both mother and infant, may be important in determining the frequency of acquisition of chlamydial infection by infants and the nature of its manifestations. Chlamydial infections in adults induce both cell-mediated and humoral immune responses (3–9). Infants acquire chlamydial IgG antibodies transplacentally (10, 11) and produce antibodies in response to postnatal infection (11, 12). Cell-mediated immune responses of infected and uninfected mothers and infants to chlamydiae, however, have not been investigated. This study examines cell-mediated immune responses to chlamydial antigens in infected and uninfected women during pregnancy and postpartum, and in infected and uninfected infants during the neonatal period.

Patients, Materials, and Methods. *Study population.* The pregnant women studied were enrolled in a prenatal clinic that serves a predominantly urban, low income population. Infected and uninfected women were identified by cervical cultures for *Chlamydia trachomatis* and were classified as seropositive or seronegative according to the presence or absence of anti-chlamydial IgG serum antibodies. Initial tests were usually carried out during the second trimester of pregnancy. At the next monthly visit, permissions to collect blood samples for assessment of chlamydial cell-mediated immunity (CMI) were requested from chlamydia-infected, seropositive women and from an approximately equal number of uninfected, seronegative women. For studies of chlamydial CMI in infants of chlamydia-infected and uninfected mothers during the neonatal period (first 4 weeks of life), blood was collected at the time of delivery from the fetal side of the placenta (umbilical cord blood) and by venipuncture from infants at the first

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visit to the pediatrician which was usually at 3 to 4 weeks of age. Conjunctival and nasopharyngeal chlamydial cultures from infants, and maternal blood specimens for assessment of chlamydial CMI in postpartum women were also obtained at this visit. In addition to specimens from these patients, blood samples were collected from infants who were brought to the pediatric outpatient department during the neonatal period because of purulent conjunctivitis that was later proved by culture to be caused by *C. trachomatis.* Blood specimens were also obtained from the mothers of these infants because they were presumed to have had chlamydial infection during pregnancy.

Written informed consent for conjunctival and nasopharyngeal chlamydial cultures and venous blood specimens from infants was obtained from parents or guardians. Oral consent for venous blood samples was obtained from adults. Cervical swabs for chylamydial cultures were obtained from pregnant women as part of the screening procedure for detection of genital infection by *Neisseria gonorrhoeae.* Umbilical cord blood specimens from placentas consisted of material that is ordinarily discarded. The study protocol was approved by the hospital's Institutional Review Board for Human Investigation.

Specimen collection and culture for chlamydiae. Endocervical swabs from pregnant women and conjunctival swabs and nasopharyngeal aspirates from infants were tested for *C. trachomatis* in cultures of McCoy cells by a modification of the method of Ripa and Mårdh (13, 14). Chlamydial inclusions were detected by iodine staining.

Serology. An enzyme-linked immunosorbent assay (ELISA) (Whittaker MA Bioproducts, Inc., Walkersville, Md.) was used to detect IgG antibodies against *C. trachomatis.* Plasma samples were used when antibody determinations were performed simultaneously with assays of the lymphocyte proliferative response (LPR) because preliminary testing of paired serum and heparinized plasma samples from 10 patients revealed only insignificant differences between amounts of antibody detected in serum and plasma. The absorbance of the hydrolyzed substrate was read in an MR-600 Dynatech microplate reader (Dynatech). Readings were recorded as optical density units which were translated into chlamydial antibody units by a linear regression plot. Values >0.16 units were considered to indicate the presence of specific anti-chlamydial antibodies. Values ≤0.16 units were considered to represent nonspecific reactivity. This limiting value was determined by the manufacturer by calculating the mean + 2.5 SD value for 200 serum samples which were found to be negative for chlamydial antibodies by microimmunofluorescence.

Cytomegalovirus antibodies were detected by an immunofluorescence assay (15, 16). Viral IgM antibodies were measured using a monospecific anti-human IgM antibody (Zeus Scientific, Inc., Raritan, N.J.). In this system an IgM titer ≥1:8 is considered to be indicative of a recent cytomegalovirus infection (15).

Preparation of chlamydial antigens for lymphocyte proliferation assays. The antigen preparation of *C. trachomatis,* immunotype E, was derived from a human urogenital isolate (UW-5/Cx), kindly supplied by S.-P. Wang, the University of Washington. The medium was removed from flasks containing 72-hr-old McCoy cell monolayers (2×10^7 cells per 150 cm^2 T flask) and was replaced with 3.0 ml of diethylaminoethyl-dextran (DEAE-dextran) 30 μg/ml. Following a 20-min incubation at 25°C, the DEAE-dextran was removed and 1.0 ml of a semi-crude preparation of *C. trachomatis* elementary bodies (EB) was added to the cell cultures. The titer of the inoculum was previously adjusted to produce a 90–100% infection in the McCoy cell monolayers. The flasks were tilted every 20 min during the 2-hr adsorption period to distribute the inoculum evenly over the cells. Fifty milliliters of fresh Eagle's minimal essential medium (MEM) containing Hanks' BSS, 10% heated (56°C, 30 min) fetal calf serum, 0.03 m*M* glucose, 0.5 μg cycloheximide, and 0.0016 m*M* glutamine per milliliter (MEM) was added to the infected McCoy cells and the flasks were reincubated at 35°C for 72–96 hr.

For harvest of the chlamydiae, McCoy cells were scraped from the flasks with rubber policemen, transferred to tubes containing twelve 3-mm glass beads (Fisher), and sonicated for 5 min in an ultrasonic bath (Heat Systems Ultrasonics, Inc.) containing ice water. The lysate was centrifuged at 650*g* for 10 min to remove cell debris and nuclei. The supernatant was then centrifuged at 10,400*g* for 40 min to pellet

the chlamydial EB. This semi-crude preparation of EB was resuspended in 2× sucrose–phosphate–glutamine, placed in a petri dish, positioned 76 cm below a uv light source, and irradiated for 15 min. Uninfected McCoy cells, processed in an identical manner, were used as a control in lymphocyte proliferation assays.

C. trachomatis, immunotype LGV-2 (LGV-2) was prepared from strain 434 supplied by H. D. Caldwell, the University of Washington. LGV-2 was grown in monolayer cultures of McCoy cells prepared in 150-cm^2 flasks as previously described, except that preinoculation treatment with DEAE-dextran was not done. After harvest and sonication of the infected cells, the lysate was centrifuged at 850*g* for 10 min. The pellet of cell debris was discarded and after uv inactivation, the supernatant was used as LGV-2 antigen. Uninfected McCoy cells prepared in an identical manner were used as a control.

After uv irradiation, aliquots of each antigen were inoculated into McCoy cell cultures to assure that these preparations contained no infectious organisms.

Lymphocyte proliferation assay. Fractions of mononuclear cells containing 85 to 95% lymphocytes were isolated from heparinized peripheral or cord blood by Ficoll–Hypaque centrifugation. The methods used for lymphocyte cultures were similar to those employed in previous studies of the lymphocyte proliferative response to whole antibiotic-killed bacteria in our laboratory (17). Lymphocyte microcultures contained 10^5 cells per 0.1 ml of RPMI 1640 medium supplemented with 25 m*M* Hepes (*N*-2-hydroxythehylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Microbiological Associates, Bethesda, Md.), penicillin (25,000 units/100 ml), and streptomycin (10 mg/100 ml), and 20% heat-inactivated autologous plasma. Preliminary tests showed that the LPR was not affected by the presence or absence of chlamydial antibody in plasma added to the test system. Cultures were performed in 96-well flat-bottomed microculture plates (No. 25860, Corning Glass Works, Corning, N.Y.). Five hours before the end of the period of incubation of lymphocytes with antigen, 0.5 μCi of [*methyl*-^{3}H]thymidine (sp act 23 Ci/mmole) was added to each culture in a 10 μl vol. Cells were harvested with a lymphocyte microharvester (Otto Hiller Co., Madison, Wis.) and incorporation of tritiated thymidine was measured using a Searle Isocap 300 liquid scintillation counter. The LPR was expressed as the mean net difference between the highest response to the antigen and the highest response to McCoy cell control material in triplicate cultures. Responses to McCoy cell control material were never lower than background thymidine incorporation in unstimulated culture assays.

The kinetics and dose response curves of LPRs to chlamydial antigens were studied in nine, nonpregnant female laboratory workers. For assessment of kinetics, lymphocyte cultures were exposed to a single optimal dilution of antigen. Cultures were then pulsed and harvested after serial incubation periods of 2 to 7 days. To determine the antigen concentration which would induce maximal LPRs, lymphocytes were exposed to several dilutions of chlamydial antigens. LPRs were assayed after a 5-day incubation period. Lymphocytes from both high and low responders were tested. On the basis of the results of kinetic and dose response studies of lymphocytes obtained from these laboratory workers, 5 days was selected as the incubation period for LPR assays in mothers and infants. Ten microliters of 1:10, 1:20, 1:50, and 1:100 dilutions of antigen were added to lymphocyte cultures in triplicate.

Lymphocyte responsiveness to mumps antigen, streptolysin O, and chlamydial antigens were assessed in parallel (18–20). Mumps antigen was prepared by dialyzing mumps skin-testing antigen (Eli Lilly) against 8 liters of PBS, pH 7.4 (21). Streptolysin O (Fisher Diagnostics) was diluted in distilled water and dialyzed. Four concentrations of each antigen were used for each study. These concentrations were selected on the basis of their ability to produce maximal LPRs in preliminary determinations of dose responses with a wide range of antigen concentrations. The reactivity of lymphocytes was also tested against four concentrations of the mitogen, phytohemagglutinin (PHA) (Difco Labs, Detroit, Mich.). Cultures containing PHA were incubated for 3 days and those containing antigen for 5 days.

Statistical methods. For each series of dilutions of chlamydial, streptococcal, and mumps antigens and of PHA, the highest mean response of each patient was used for statistical analysis. The differences between

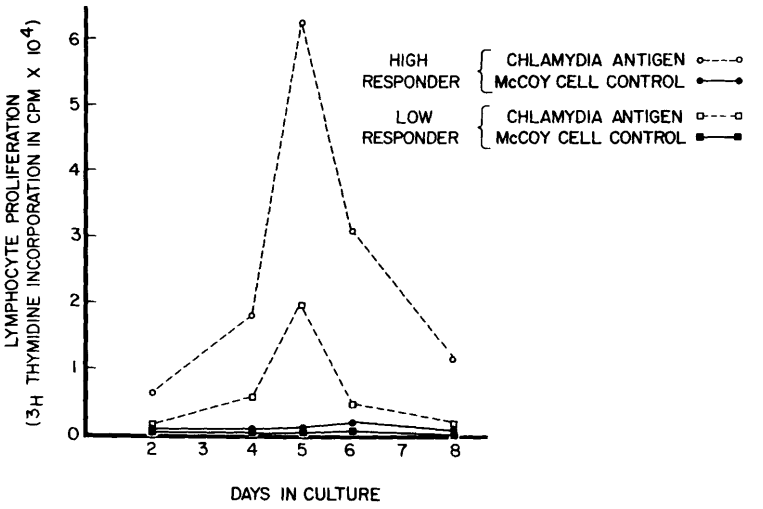

FIG. 1. Kinetics of the lymphocyte proliferative response to *Chlamydia trachomatis* serotype E antigen. The high and low responders shown in this figure were arbitrarily defined on the basis of their responses to optimal concentrations of chlamydial antigen.

means of groups were analyzed by Student's *t* test for unpaired variables. Correlation coefficients for serological results were calculated and analyzed using standard statistical methods.

Results. *Kinetics and dose response curves of LPRs to chlamydial antigens.* LPRs to an optimal dilution of antigen prepared from *Chlamydia trachomatis,* serotype E, were observed to be maximal after an incubation period of 5 days (Fig. 1). Therefore, 5 days was selected as the incubation period for all LPR assays. In tests of varying dilutions of antigen, maximal lymphocyte stimulation occurred after incubation with antigen dilutions of 1:10, 1:20, 1:50, and 1:100 (Fig. 2). Therefore, in each assay, lymphocyte cultures in triplicate were incubated with each of these dilutions of

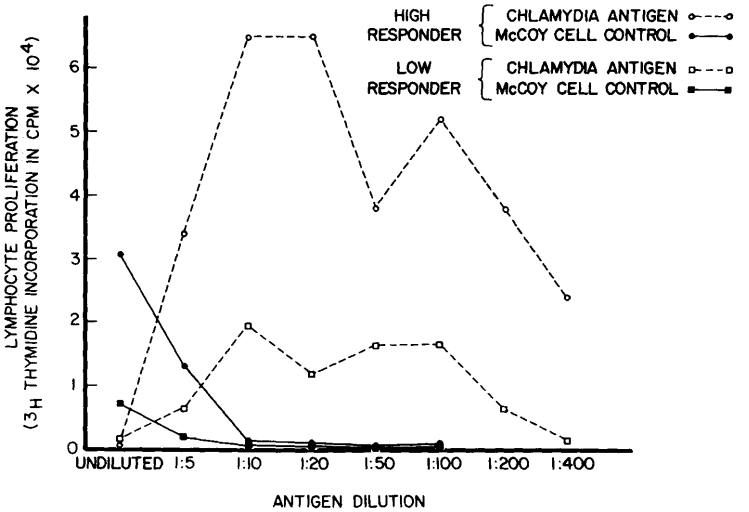

FIG. 2. Effect of antigen concentration on the lymphocyte proliferative response to *Chlamydia trachomatis* serotype E in two persons arbitrarily defined as high and low responders on the basis of differences in [*methyl*-^{3}H]thymidine incorporation between cultures exposed to chlamydial antigen or McCoy cell control.

antigen. As shown in Figs. 1 and 2 background counts in cultures exposed to McCoy cell control material were low. These background counts are representative of those observed throughout the study.

LPRs of chlamydia-infected and uninfected pregnant and postpartum women to antigens of C. trachomatis. Pregnant women were classified as infected if *C. trachomatis* was isolated from the cervix at any time during pregnancy. Postpartum women were considered to be infected if this organism had been isolated from the cervix during pregnancy or was isolated from their infants during the first month of life. All infected women were seropositive for chlamydial IgG antibodies. Women who were seronegative and whose cervical cultures taken during pregnancy failed to grow *C. trachomatis* were considered to be uninfected. Table I compares the LPRs of infected and uninfected pregnant and postpartum women to chlamydial and control antigens and to PHA. Both during pregnancy and in the postpartum period, mean LPRs to *C. trachomatis* immunotype E and LGV-2 antigens were greater in infected women than in uninfected women ($P < 0.01$). The mean LPRs of infected women to these antigens were greater during the postpartum period than during pregnancy ($P < 0.001$). There were no statistically significant differences among the responses of infected and uninfected women to mumps antigen, streptolysin O, and PHA, either during pregnancy or postpartum.

LPRs of infected and uninfected infants. LPR assays on infants whose mothers were studied during pregnancy were performed either on umbilical cord blood obtained at birth or on venous blood obtained at 2–4 weeks of age. Chlamydial cultures to correspond with cord blood samples were not obtained because we had found in a previous study that detection of *C. trachomatis* at 3 days of age was infrequent, even in infants from whom the organism was isolated later (22). Conjunctival and nasopharyngeal cultures for *C. trachomatis* were obtained from all infants at the same time as the venous blood samples. Cultures of infants born to both infected and uninfected mothers were negative and none of these infants developed conjunctivitis or pneumonia. The infected infants who were studied were patients in whom chlamydial conjunctivitis

TABLE I. LYMPHOCYTE PROLIFERATIVE RESPONSES TO ANTIGENS OF *Chlamydia trachomatis*, CONTROL ANTIGENS, AND PHYTOHEMAGGLUTININ IN CHLAMYDIA-INFECTED AND UNINFECTED PREGNANT AND POSTPARTUM WOMEN

Patient category	No. tested	Antigens[a]				
		C. trachomatis immunotype E	LGV-2	Mumps	Streptolysin	Phytohemagglutinin
Pregnant women						
Uninfected, seronegative	11	3,029 ± 1,066[b]	1,200 ± 541[b]	14,000 ± 2,924	49,320 ± 14,856	101,822 ± 26,101
Infected, seropositive	18	15,599 ± 2,843[b,d]	10,896 ± 2,785[b,d]	26,879 ± 6,339	43,974 ± 10,371	149,945 ± 18,498
Postpartum women						
Uninfected, seronegative	9	5,517 ± 1,802[c]	4,609 ± 1,819[c]	30,585 ± 6,055	47,780 ± 16,088	148,283 ± 12,689
Infected, seropositive	20	33,188 ± 5,403[c,d]	25,077 ± 4,295[c,d]	30,095 ± 3,184	48,697 ± 6,005	125,833 ± 10,687

[a] Lymphocyte proliferative responses to antigens are expressed as mean cpm ± SE above responses to control material.
[b] Differences between responses of uninfected and infected pregnant women are statistically significant ($P < 0.01$).
[c] Differences between responses of uninfected and infected postpartum women are statistically significant ($P < 0.001$).
[d] Differences between responses of infected, seropositive women during pregnancy and postpartum are statistically significant ($P < 0.05$).

was confirmed by culture in the pediatric ambulatory clinic.

The LPRs of infants to antigens of *C. trachomatis,* control antigens, and PHA are presented in Table II. The intense LPRs to the mitogen, PHA, demonstrate that lymphocytes from all infants tested were capable of responding when appropriately stimulated. At birth the mean LPR of umbilical cord blood samples from infants of infected mothers to antigen prepared from *C. trachomatis* immunotype E was greater than that of infants born to uninfected mothers ($P < 0.05$). At 2–4 weeks of age this difference persisted but was not statistically significant because of the large SE of the mean LPR of older infants of infected mothers. At the same age, there was a statistically significant difference ($P < 0.005$) between the mean chlamydial LPRs of infants with chlamydial conjunctivitis and of uninfected infants of uninfected mothers. There were no statistically significant differences between mean chlamydial LPRs of infants with conjunctivitis and of uninfected infants of infected mothers, or among mean LPRs of different categories of infants to mumps antigen, streptolysin O, or PHA.

Cytomegalovirus antibodies. Cytomegalovirus IgM antibodies were not detected in the sera of any of the infants with chlamydial conjunctivitis. Antibodies were detected in a titer of 1:16 in the serum of one of their mothers.

Discussion. Chlamydial infections have been shown to induce cell-mediated immune responses in nonpregnant adults (3, 5–9). In this study LPRs to chlamydial antigens were greater in infected than in uninfected pregnant women, but were lower during pregnancy than during the postpartum period. This suggests that CMI against chlamydiae is depressed during pregnancy. This finding is consistent with those of other investigators who have reported depression of CMI against chlamydiae and other microorganisms during pregnancy (23–25). Additional studies are needed to determine whether or not depression of CMI against chlamydiae during pregnancy results in increased susceptibility of pregnant women to recurrent or chronic genital infection with *C. trachomatis.*

Chlamydial IgG antibodies are known to be acquired transplacentally by infants in amounts proportional to levels of circulating maternal antibodies (11). The finding of increased LPRs to chlamydiae in umbilical cord bloods of infants of chlamydia-infected mothers, however, was unexpected. Presently available evidence indicates that chlamydial infection of the newborn occurs during delivery and that at least several days of replication in infected anatomic sites are required for concentrations of the organisms to reach levels that are detectable by culture (12, 22). In a recent survey, *C. trachomatis* was not detected in cultures of 801 placentas (26). This finding further supports the concept that chlamydial infections during pregnancy remain confined to the lower genitourinary tract. Prior intrauterine infection, therefore, is an unlikely cause of the increased lymphocyte response to chlamydial antigens in umbilical cord bloods of these infants. Another possible cause of this response is contamination of test antigens by mitogens to which the lymphocytes of most newborns react vigorously (27). However, the low responses of umbilical cord blood lymphocytes from infants of uninfected, seronegative mothers to chlamydial antigens indicate that our antigen preparations do not contain or act as mitogens. A more likely possibility is that maternal CMI against chlamydiae is passively transferred to the fetus. Maternal–fetal transfer has been suggested as the mechanism for neonatal CMI to mycobacterial purified protein derivatives (28), streptokinase/streptodornase, and candida species (29). Our surveillance for chlamydial infections has resulted in initiation of a treatment program for the population of pregnant women under study. Infants born to mothers whose recent chlamydial infections were eradicated by treatment prior to labor and delivery will be tested for lymphocyte responsiveness to chlamydial antigens. The results of these tests are expected to aid in determining whether chlamydial CMI in infants is acquired transplacentally or requires direct exposure to *C. trachomatis.*

At 2–4 weeks of age, vigorous LPRs to chlamydial antigens were observed both in infants with chlamydial conjunctivitis and in uninfected infants of infected mothers. In the latter group there was a wide range in intensity of chlamydial LPRs but in infants with conjunctivitis, LPRs were uniformly at high levels. This suggests that in some of these exposed

TABLE II. LYMPHOCYTE PROLIFERATIVE RESPONSES TO ANTIGENS OF *Chlamydia trachomatis*, CONTROL ANTIGENS, AND PHYTOHEMAGGLUTININ IN INFANTS DURING THE NEONATAL PERIOD

Category of infants	No. tested	Antigen[a]				
		C. trachomatis immunotype E	LGV-2	Mumps	Streptolysin	Phytohemagglutinin
Infants at birth						
Mother uninfected	14	3,030 ± 1,091[b]	2,461 ± 520	7,209 ± 2,202	18,994 ± 11,087	139,533 ± 15,579
Mother infected	13	8,740 ± 2,236[b]	3,026 ± 912	5,916 ± 2,146	22,787 ± 17,610	101,046 ± 14,631
Infants 2–4 weeks of age						
Uninfected infants of uninfected mothers	9	7,754 ± 3,065[c]	3,370 ± 1,110[c]	10,421 ± 3,178	24,207 ± 15,371	147,819 ± 10,823
Uninfected infants of infected mothers	10	22,278 ± 9,412[d]	7,894 ± 3,070[d]	8,872 ± 2,272	17,673 ± 6,315	107,929 ± 18,692
Infants with chlamydial conjunctivitis	9	26,005 ± 4,238[c]	13,492 ± 2,573[c]	9,788 ± 2,324	24,865 ± 13,414	127,690 ± 27,850

[a] Lymphocyte proliferative responses to antigens are expressed as means cpm ± SE above responses to control materials.
[b] Differences between responses of infants of uninfected and infected mothers are statistically significant ($P < 0.05$).
[c] Differences between responses of uninfected infants of uninfected mothers and infants with chlamydial conjunctivitis are statistically significant ($P < 0.005$).
[d] Differences between responses of uninfected infants of infected mothers and both uninfected infants of uninfected mothers and infants with chlamydial conjunctivitis are not statistically significant.

but culture negative infants, increased LPRs to chlamydiae may have resulted from infection of anatomic sites, such as the rectum, that were not cultured. In an ongoing prospective study of infants born to chlamydia-infected mothers, Grossman *et al.* found infection rates of 34 and 62% as detected by serial cultures and seroconversion, respectively (30). Therefore, chlamydial infections are not always detected by culture, even when frequent serial cultures of multiple sites are obtained. In studies by other investigators of maternal–infant chlamydial infections in monkeys, infection was not detected by serial cultures of infant monkeys born to mothers with cervical infection (31). When the conjunctivae of these infant monkeys were inoculated with live *C. trachomatis* at age 7 months, however, infants of infected mothers developed a more severe conjunctivitis than infants of uninfected control monkeys. It was postulated that prior sensitization of the infant monkeys of infected mothers had occurred, possibly from undetected infection acquired at birth. However, our observations of increased LPRs to chlamydial antigens in umbilical cord bloods and later in exposed but apparently uninfected human infants suggest prenatal passive transfer of CMI as an alternative explanation for the hypersensitivity to chlamydiae observed in these infant monkeys. The hypothesis that induction of chlamydial CMI may involve mechanisms other than, or in addition to, infection of mucosal sites is further supported by the observation that chlamydial ocular infections in monkeys failed to induce CMI, except in animals that received intensive prior immunization with chlamydiae (32).

The role of immune responses in resolution of chlamydial infections and resistance to reinfection is poorly understood. Neither transplacentally acquired nor actively induced chlamydial antibodies appear to be protective against infection, and both protective and sensitizing roles have been ascribed to chlamydial CMI. In trachoma, chlamydial CMI has been postulated to contribute to tissue injury (33). In some animal models of systemic chlamydial infections, however, CMI has been reported to be an important defense mechanism (34–37). Although the detection of lymphocyte proliferation in response to stimulation by chlamydial antigens does not prove that protective CMI has been induced, lymphocyte proliferation in response to antigenic stimulation is usually indicative of concurrent development of other manifestations of CMI such as lymphokine production and delayed hypersensitivity. The role of these aspects of the immune response in protection against or resolution of chlamydial infections requires investigation.

Chlamydial pneumonia occurs later in infancy and much less frequently than conjunctivitis (2, 22, 38). Factors that determine whether or not pneumonia develops during the natural course of neonatally acquired chlamydial infections may include synergistic or immunosuppressive effects of concomitant respiratory infections with other pathogens such as cytomegalovirus (39) and variations in rates of maturation of the immune system. None of the infants with conjunctivitis in this study developed pneumonia, probably because all were treated with antimicrobials and were monitored to ensure eradication of both conjunctival and nasopharyngeal infections (14). In addition, none of the infants and only one of their mothers has serologic evidence of cytomegalovirus infection. Whether the increased lymphocyte responsiveness detected in these infants is a protective mechanism or a potentially pathogenic factor in the natural history of this infection cannot be determined from presently available data.

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