Immunohistochemical Study of the Local Inflammatory Response to Chlamydial Ocular Infection

Judith A. Whitrum-Hudson, Hugh R. Taylor, Mahmood Farazdaghi, and Robert A. Prendergast

Immunohistochemical staining of conjunctival biopsies from cynomolgus monkeys (Macaca fascicularis) was performed after they received a single primary ocular infection, a single secondary challenge infection, or repeated ocular inoculations with Chlamydia trachomatis. T cells of the suppressor/cytotoxic (OKT8F) phenotype predominated regardless of the infection protocol, and perifollicular T lymphocytes of both the suppressor/cytotoxic and helper (OKT4A) phenotypes appeared in large numbers during the peak inflammatory reaction. In repeatedly inoculated monkeys, T cells and follicles persisted until cessation of reinfection. IgM-bearing B lymphocytes comprised the majority of cells within follicles, with smaller numbers of IgG- or IgA-positive B cells. The major difference in the response to the various infection protocols was the increased number and persistence of follicles with repeated reinoculation. The finding of large numbers of T-suppressor/cytotoxic and T-helper cells in the infected conjunctiva supports a role for cell-mediated immunity in the local response to C. trachomatis ocular infection.


Trachoma, an ocular infection with Chlamydia trachomatis, is a leading cause of blindness in many parts of the world. An animal model of trachoma has been developed in cynomolgus monkeys (Macaca fascicularis) by repeated infection with this organism. Although serum and tear antibody responses are induced readily after experimental chlamydial infections, anti-chlamydial antibody does not protect against reinfection. The role that cellular immunity may play in either protection or exacerbation of trachoma is poorly understood. Recent studies of peripheral lymphoid responses to chlamydial antigens and mitogens in cynomolgus monkeys suggest that chronic chlamydial eye infection induces suppressor T lymphocytes which may prevent the development of protective immune responses.

To date, few histological studies of local immune responses have been performed in experimental trachoma. Since the intense inflammatory response and subsequent scarring of infected conjunctiva may be a consequence of immune mechanisms rather than of direct chlamydial cytopathic effects, it is important to investigate the lymphocyte populations comprising the local inflammatory response during chlamydial ocular infection. In this communication, we report the results of immunohistochemical studies utilizing appropriate, cross-reacting, anti-human T- and B-cell reagents to examine cells in the lymphoid populations present in cynomolgus monkey conjunctiva during chlamydial infection.

Materials and Methods

Chlamydia trachomatis Infection

Two groups of five young adult male cynomolgus monkeys (Hazelton Laboratories; Alice, TX) were studied. All procedures described herein conform to the ARVO Resolution on the Use of Animals in Research. C. trachomatis was grown in McCoy cells, purified using standard procedures, and diluted in phosphate buffered saline. Each eye of the monkeys in the first group received a primary topical inoculation of 20 μl of a suspension of HAR-36, a B serovar of C. trachomatis (kindly provided by Dr. A. Bruce MacDonald, University of Massachusetts, Amherst, MA). Each eye received an inoculum containing approximately 2000 inclusion-forming units (IFU). An identical second inoculation was given to each of these eyes 18 wk later. In the second group of monkeys, both eyes were inoculated each week with the same dose of C. trachomatis for a total of 18 inoculations.

Monkeys were examined weekly and clinical findings expressed as previously described in terms of a follicular index and an inflammatory index for each experimental group. The follicular index was an aggregate score...
derived by grading on a scale of 0 (neg) to 4+ (maximum) of tarsal, superior fornix, bulbar, and limbal follicles. Similarly, an inflammatory index was obtained as an aggregate score for the severity of papillae, bulbar conjunctival injection, and ocular discharge.

**Tissue Collection**

Conjunctival biopsies were obtained at 1, 2, 5, 18, 19, 20, 23, and 27 wk following initial infection of both groups of monkeys. On each occasion, conjunctival biopsies from the superior fornix were collected from two monkeys in each group so that each monkey was biopsied at least three times. Tissue was snap frozen, embedded in OCT embedding compound (Tissue-Tek; Miles Scientific, Naperville, IL), and stored at -70°C. Six to eight micron frozen sections were cut and mounted on gelatin-coated slides (1% gelatin with 0.04 mg/100 ml chromium potassium sulfate), stored unfixed at 4°C, and stained within 1 wk.

**Immunohistochemistry**

The ABC technique of Hsu et al \(^\text{11}\) was used to stain lymphocyte subsets. T-helper cells (T\(_H\)) were stained with mouse monoclonal antibody OKT4A, and T-suppressor/cytotoxic cells (T\(_{S/CTL}\)) were stained with monoclonal antibody OKT8F (kindly provided by Dr. Gideon Goldstein, Ortho Pharmaceutical; Raritan, NJ) at a concentration of 1–2 \(\mu\)g/ml. These anti-human T-cell monoclonal antibodies have been previously reported to cross-react with primate T-cell subsets. \(^\text{12}\) Heavy chain-specific goat anti-human immunoglobulin antisera were used to stain IgM-, IgG-, or IgA-positive B lymphocytes (Kirkegaard and Perry Labs, Gaithersburg, MD). All three of these antibodies were used at a concentration of 10 \(\mu\)g/ml. Secondary antibodies and the ABC complex were used as provided in Vectastain ABC kits (mouse and goat kits for anti-T- and anti-B-cell antibodies respectively, Vector Labs, Burlingame, CA). All washes and dilutions were made in Tris-HCl buffered saline, pH 7.6.

Six to eight micron frozen sections were fixed in acetone for 5 min immediately prior to staining; all sections from any single conjunctival biopsy were stained on the same day. Sections which had primary antibodies omitted exhibited no staining; other sections stained with OKM-1 exhibited no positive cells inside follicles, and only a small number of positive cells were located between large follicles and the epithelium (fewer than 10 cells per section). The total area of each conjunctival tissue was measured in multiple sections with a Zeiss MOP Scanner (Carl Zeiss, Inc.; Thornwood, NY). The percent area of the lymphocytic infiltration including germinal centers at each time point was determined by taking the average from multiple sections obtained from each of the animals biopsied. A lymphocyte index was calculated for each animal by multiplying the graded percentage of this area occupied by histochemically positive cells. The grading of positive cells was 0 for no staining; 1+ for less than 25% staining; 2+ for 25 to 50% staining; 3+ for 50 to 75% staining; and 4+ for greater than 75% staining.

**Results**

**Follicle Development during Ocular Chlamydial Infection**

Most changes in T and B cells during infection were follicle-associated, and therefore we determined changes in number or size of follicles during the course of primary, secondary, and repeated inoculation. The percentage area of conjunctiva occupied by follicles generally paralleled the clinical response. Peak follicular development was observed 2 wk following a single inoculation with *C. trachomatis* and 1 wk following a single secondary challenge inoculation (Fig. 1a). Repeated inoculation induced more and larger follicles, with maximum follicle development occurring at 5 wk (Fig. 1b).

**Local Lymphoid Response to Primary Infection**

In normal monkeys which had not been exposed to *C. trachomatis*, follicles were observed only rarely in the upper fornix, and small numbers of scattered subepithelial T\(_{S/CTL}\) and stromal T\(_H\) were seen. When follicles were present in normal conjunctivae, IgM-positive cells predominated with smaller numbers of IgG- and IgA-bearing cells, and the perifollicular infiltrate of T cells was not observed.

Following a single inoculation with *C. trachomatis*, a mild and self-limited inclusion conjunctivitis rapidly developed, with maximum inflammation and follicular development occurring between 2 and 4 wk (Fig. 1a). Increased numbers of all five T- and B-lymphocyte subpopulations were observed between 1 and 2 wk, with the concentration of cells bearing the T\(_{S/CTL}\) phenotype always surpassing numbers of T\(_H\). Representative T-cell staining at 1+ and 3+ grading of positivity are shown in Figure 2; 4+ staining is depicted in Figure 3. B lymphocytes were found almost exclusively within the follicles which developed during chlamydial infection, whereas T cells were found primarily in a perifollicular location (Fig. 3). IgM-positive B cells represented the majority of cells within follicles, regardless of follicle size or the infection protocol (Figs. 4, 5). IgG- and IgA-positive B cells appeared to be present in equal numbers between 2 and 5 wk. By 5 wk, only T\(_H\) had returned to background levels. However, 18 wk after a primary inoculation, the follicular response
had resolved and the number of lymphocytes of each subset had returned to background levels. These data are graphically portrayed in the lymphocyte index measurement (Fig. 5).

Local Lymphoid Response to Secondary Infection

When monkeys received a secondary inoculation with C. trachomatis 18 wk after primary infection, a less severe and less protracted clinical response developed in which follicles appeared rapidly but then decreased within 2 to 3 wk (Fig. 1a). Perifollicular T_H and T_S/CTL increased rapidly to high levels within one week after secondary challenge but had returned to background levels within 5 wk (Figs. 5a-b).

The B-cell response to secondary challenge with C. trachomatis was more vigorous than that observed following primary inoculation and reflected the more marked follicular response. Within 1 wk, IgM-positive cells had increased in number more than fivefold and persisted as the dominant immunoglobulin isotype until follicles disappeared (Fig. 5c). IgG- and IgA-positive B cells appeared in greater numbers following secondary challenge, but these were delayed approximately one week compared to IgM-positive cells (Figs. 5d-e). Since the increase in IgG- and IgA-positive cells did not correlate with any substantial decrease in IgM-positive cells, the possibility of co-expression of IgM with IgG or IgA on some B cells cannot be ruled out in these experiments. Staining performed with two monoclonal, non-immunoglobulin specific, anti-human B cell antibodies (Miles Scientific and Ortho Pharmaceutical) showed staining entirely limited to follicles. Similarly, goat anti-human IgG, IgM, and IgA antibodies stained only follicular cells.

Fig. 1. The clinical response and histologic assessment of follicle development in cynomolgus monkeys following ocular inoculation with C. trachomatis. The follicular index (solid circles) and an inflammatory index (open circles) are shown for each group, and bars depict the percent area of the conjunctiva occupied by follicles. (a) Single primary and secondary inoculation; (b) Repeated inoculation. There were five monkeys in each group.

Fig. 2. Representative staining of T cells with OKT8F: (a) 1+ staining seen at 2 wk post-inoculation; (b) 3+ staining at 4 wk of repeated inoculation. Similar ranges of staining were seen for OKT4A-positive cells, but maximal infiltration involved fewer OKT4A- than OKT8F-positive cells (compare with 4+ staining in Fig. 4). Sections were counterstained with methyl green; final magnification is X230.
Chronic Infection

Repeated weekly inoculation with *C. trachomatis* induced chronic follicular chlamydial infection (tra-

Fig. 3. Representative staining of T lymphocytes in frozen sections of monkey conjunctiva: (a) T-helper cells (OKT4A), (b) T-suppressor/cytotoxic cells (OKT8F), (c) negative control for T-cell staining. Sections were counterstained with methyl green. Final magnification is ×86; the bar represents 100 microns.

Fig. 4. Representative staining of B lymphocytes in frozen sections of monkey conjunctiva: (a) IgM-positive B cells, (b) IgG-positive B cells, and (c) IgA-positive B cells. Sections were counterstained with methyl green. Final magnification is ×205; the bar represents 100 microns.
Fig. 5. The proportion of lymphocyte subsets (lymphocyte index) in frozen sections of monkey conjunctivae stained by the ABC method for the presence of (a) T-helper cells, (b) T-suppressor/cytotoxic cells, (c) IgM-positive B cells, (d) IgG-positive B cells, and (e) IgA-positive B cells. Monkeys which received a single primary inoculation and then a single secondary challenge at 18 wk (open bars) are compared with monkeys which received 18 weekly repeated inoculations (closed bars) of *C. trachomatis*. Each bar represents mean results of biopsies from two monkeys.

In the monkeys of the second experimental group, follicles developed rapidly and increased in size over 4 to 6 wk, persisting until repeated inoculation was stopped at 18 wk (Fig. 1b), after which the clinical disease waned over the succeeding 4 wk.

More T cells were seen in the conjunctivae of these animals (Figs. 5a–b) than were observed following either primary or secondary infection. The highest levels of TH and TS/CTL were reached after 5 wk of repeated inoculation and then decreased in proportion to the decline in follicle size when repeated inoculation was stopped. Numbers of TS/CTL greatly surpassed numbers of TH throughout the experimental period. During active infection, TH were found in the perifollicular regions in contrast to their deeper location in normal conjunctiva. When reinoculation was stopped, the number of TS/CTL and TH fell quickly but some TS/CTL remained in the subepithelium after follicles had disappeared. Only rare T cells were observed within the epithelium in either experimental group. In areas of thinned epithelium overlying follicles, numerous T cells were present and could not be distinguished from the perifollicular infiltrate. This observation is consistent with our earlier histopathologic studies.1

Immunoglobulin-bearing B cells appeared in conjunction with the vigorous follicle development seen during repeated inoculation (Figs. 5c–e). Peak levels of all three isotypes were seen at 5 wk though IgM-positive cells predominated. Although the total number of IgA- and IgG-positive cells fell when repeated inoculation ceased, high levels of IgM-positive cells persisted for 2 wk longer. All three subpopulations of B cells declined to background levels within 2 to 5 wk.

Discussion

Follicles, or germinal centers, are the hallmark of trachomatous inflammation. Changes in conjunctival lymphocyte subpopulations associated with follicles were studied during primary and secondary single infection and repeated inoculation with *C. trachomatis* in an experimental cynomolgus monkey model (trachoma). In each case there was a predominance of perifollicular suppressor/cytotoxic T cells which were found in large numbers. It has been suggested previously that cell-mediated immunity may play an important role in the pathogenesis of trachoma,7,9 and the high number of T cells in more severe disease supports this notion. The kinetics of the appearance and decline of both perifollicular TH and TS/CTL were similar, but quantitatively TS/CTL were present in at least four times the number of TH. The large number of TS/CTL and their predominance was unexpected and is unusual. A predominance of TH cells has been reported in human conjunctivae from patients with conjunctival inflammation from a variety of causes, with the exception of graft-versus-host disease where TS/CTL predominated.13 Although cells staining with OKT8F could be either suppressor or cytotoxic T lymphocytes, the lack of necrosis in conjunctivae during chlamydial infection suggests a lack of cytotoxic effector T cells. It is possible, however, that cytotoxic T cells might be responsible for extensive scarring late in trachoma either by a direct action on infected cells or by nonspecific innocent bystander effects. Regardless of their function, the predominance of TS/CTL in the conjunctiva might help explain why neither monkey nor man is solidly...
immune to reinfection with chlamydia. It is interesting to note that only small numbers of macrophages were seen and these were not associated with follicles. In some granulomatous disease states, schistosomiasis for example, monokines have been strongly implicated in the induction of fibrosis.14

IgM-positive B cells were predominant and persisted in follicles throughout the course of infection. One might have expected a shift to an immunoglobulin isotype more typical of an active mucosal immune response (eg, to IgA or IgG). We have not examined coexpression of IgM with other immunoglobulin isotypes, although previous studies of tear antibody suggest that a shift from IgM to IgG or IgA chlamydia-specific antibody occurs by 3 wk.2 In the current study, the antigen specificity of the various follicular B-cell subpopulations for chlamydia antigens was not determined. The predominance of IgM-positive cells may be an in vivo reflection of polyclonal B-cell activation since *C. trachomatis* (L2 biovar) has been shown to cause polyclonal activation of B cells in vitro.15 Alternatively, it may reflect a T-independent response to *C. trachomatis*, accounting for the observed predomiance of T<sub>S/CTL</sub> in infected conjunctivae. Functional analysis of these lymphocyte subsets is being undertaken and will aid in distinguishing between these and other possibilities.

In summary, our studies have demonstrated that there is a vigorous T- and B-lymphocyte response in the conjunctiva during chlamydial infection. Importantly, T<sub>S/CTL</sub> (OKT8F-positive cells) were far more numerous than T<sub>H</sub> (OKT4A-positive cells) and were mainly perifollicular and subepithelial. The large increase in T lymphocytes, and particularly of the T<sub>S/CTL</sub> phenotype, indicates that the local cell-mediated reactions may well be an important component in the response to chlamydial infection.

**Key words:** trachoma, cymolongus monkeys, cellular immunity, conjunctivitis, T suppressor cells

**References**