INFECTION AND IMMUNITY, Dec. 1992, p. 5294–5297
0019-9567/92/125294-04$02.00/0
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Vol. 60, No. 12

Frequency of Antigen-Specific B Cells during Experimental Ocular *Chlamydia trachomatis* Infection

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Received 13 December 1991/Accepted 17 September 1992

Chlamydia-specific antibody-secreting cells have been identified in conjunctiva and draining cervical lymph nodes by an ELISPOT assay in a cynomolgus monkey model of trachoma. These local sites contained numbers of chlamydia-specific B cells that were higher than those in distant inguinal lymph nodes and peripheral blood. The numbers of chlamydia-specific immunoglobulin G-secreting B cells observed were 5 to 57 per 10^6 cells in conjunctiva and 24 to 996 per 10^6 cells in cervical lymph nodes during conjunctival infection or after challenge of immune monkeys with the chlamydial 57-kDa heat shock protein (hsp60). These studies demonstrate a large chlamydia-specific B-cell component in the conjunctiva during ocular chlamydial infection. These results are similar to our findings for chlamydia-specific T-cell responses.

Trachoma is a major cause of preventable blindness (2, 3) and results from repeated ocular infection with *Chlamydia trachomatis*. In developing countries, it has been estimated that 350 million people are affected by this disease (3, 8). Studies of antichlamydial immunity have been important for increasing our understanding of the pathogenesis of this disease and for antichlamydial vaccine development (4, 5, 7, 11, 15, 17–19, 22). The cynomolgus monkey model of trachoma very closely parallels human ocular chlamydial infection (2, 13, 16). Large numbers of T and B lymphocytes appear in the inflamed conjunctiva (19, 21). Recently we reported that high numbers of chlamydia-specific T cells were present in this infiltrate (6). Although B-cell-containing conjunctival follicles are a hallmark of trachoma, the antigen specificity of these B cells has not been fully elucidated.

Mucosal antibody responses have been shown in other animal models to suppress acute disease and to confer partial protection from reinfection with chlamydiae (4, 7). Descriptions of the local cellular events underlying the humoral response during ocular chlamydial infection are limited. The B-cell response in ocular chlamydial infection has been largely characterized by measurement of specific immunoglobulin G (IgG) and IgA antibodies in serum and, less often, in tears during clinical and experimental ocular infection (4–8, 20). B-cell responses in chlamydial infections of other mucosal tissues have been described almost exclusively in terms of serum antibody (7). Recently, we demonstrated the in vitro production of antichlamydial antibody by isolated conjunctival lymphocytes obtained from infected cynomolgus monkeys (20). In the present study, we adapted the ELISPOT technique to directly enumerate chlamydia-specific B cells in conjunctiva and regional draining cervical lymph nodes (CLN) during ocular chlamydial infection.

C. trachomatis serovar C (TW/3) was grown in HeLa cells, and elementary bodies (EB) were purified by discontinuous density gradient centrifugation on Percoll (1). Purified EB were used as the antigen in the ELISPOT assay and as the infection inoculum. The amount of protein in the EB preparation was estimated by the standard bicinchoninic acid (BCA) protein assay method (Pierce, Rockford, Ill.). Control antigens were purified whole cholera toxin (List Biological Laboratories, Inc., Campbell, Calif.) (positive control) and thyroglobulin (Sigma Chemical Co., St. Louis, Mo.) (negative control).

Adult cynomolgus monkeys (*Macaca fascicularis*) were obtained from Charles River Primates Inc. (Port Washington, N.Y.). All procedures were performed with the monkeys under either Ketaset or gas anesthesia in accordance with the National Institutes of Health guidelines for experimental animals. The monkeys in this study had been used previously in a vaccine study during which they were immunized either with synthetically prepared polypeptide segments of the major outer membrane protein from serovar C conjugated to cholera toxin or with cholera toxin alone and then given an ocular challenge with live serovar C EB (16a). Since the vaccination did not alter responses to chlamydial challenge and the immunized animals exhibited immunologic and clinical responses identical to those of naive (unimmunized) animals, monkeys were randomized for the current experiments. Twenty weeks after the primary ocular infection and when all signs of infection had cleared, all monkeys received a secondary ocular challenge with live *C. trachomatis* serovar C (2,000 or 500 inclusion-forming units per eye). Tissues were removed 14 or 21 days postchallenge during the peak inflammatory response.

An additional group of previously infected but clinically normal ocularly immune monkeys were topically challenged with 20 μl of Triton X-100 chlamydial antigen extract (kindly provided by H. Caldwell and R. Morrison [Rocky Mountain Laboratories, Hamilton, Mont.]). The Triton X-100 was removed from the extract by passage over an Extracti-gel column (Pierce) and contained the chlamydial protein hsp60

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(1 mg/ml) (12, 17). This treatment elicits a vigorous local inflammatory response that peaks at 24 to 48 h (5, 6, 14, 17) and induces the appearance of sufficient T and B lymphocytes in conjunctivae of immune monkeys to isolate for ELISPOT or other assays. Single-cell suspensions of conjunctival lymphocytes were obtained from tissue biopsies by 0.2% collagenase (Worthington, Freehold, N.J.) digestion for 3 h in Hanks' balanced salt solution (20). Conjunctival cells were resuspended at a concentration of 5×10^5/ml.

CLN were surgically removed from the right side of deeply anesthetized monkeys before secondary infection (day 0). Additional CLN were similarly removed from two other monkeys each on days 14 and 21 after secondary challenge. Lymph nodes from four naive monkeys belonging to other investigators were also obtained. To obtain single-cell suspensions of lymph node cells, lymph nodes in Hanks' balanced salt solution containing gentamicin (10 μg/ml; Whittaker M.A. Bioproducts, Inc., Walkersville, Md.), streptomycin (125 μg/ml; Sigma), and amphotericin B (Fungizone; 0.25 μg/ml; GIBCO) were pressed through a sterile 60-mesh screen. After two washes in Hanks' balanced salt solution, the cells were suspended in RPMI 1640 containing 10% fetal bovine serum, 2 mM L-glutamine (GIBCO), 3×10^{-5} M 2-mercaptoethanol, and streptomycin (125 μg/ml). Peripheral blood (5 to 10 ml) was collected from some animals at the same time that lymph nodes were obtained. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation as previously described (20).

The ELISPOT assay was modified from that of Sedgwick and Holt (10) for use with chlamydiae and cholera toxin. Antigens (1 mg/ml) in bicarbonate-carbonate buffer (0.05 M; pH 9.8) were added to duplicate 16-mm wells of 24-well plates (no. 3524; Costar, Cambridge, Mass.) in a volume of 0.4 ml per well. The plates were incubated at 4°C overnight. The next day, the wells were washed three times with phosphate-buffered saline (PBS) and incubated with 1 ml of PBS–1% bovine serum albumin at 37°C for 1 h to block nonspecific binding sites. After blocking, 1 ml of culture medium containing 0.5×10^6 to 1×10^6 lymph node or conjunctival cells was added to each well and the plates were incubated for 2 h in 5% CO_2 in a humidified incubator. The plates were washed thoroughly with PBS containing 0.02% Tween (Sigma) (PBS-Tween) to remove all cells. Then 0.5 ml of alkaline phosphatase-conjugated goat anti-human IgG, IgM (1:500), or IgA (1:250) (KPL, Gaithersburg, Md.) in PBS-Tween containing bovine serum albumin was added to each well, and the plates were stored overnight in the dark at room temperature. The next day, the wells were washed three times with PBS-Tween, and 0.5 ml of substrate solution (5-bromo-4-chloro-3-indolyl phosphate dissolved in 2-amino-2-methyl-1-propanol buffer) at a final concentration of 0.05% in 0.6% agarose was added to each well (9). After the agarose overlay solidified, the plates were incubated for 1 h at 37°C. Spots were enumerated by using an inverted microscope (magnification, ×4), and results are expressed as the mean numbers of spot-forming cells (SFC) per 10^6 cells from at least two wells per antigen.

Conjunctival biopsies were taken either from actively infected monkeys or from the ocularly immune monkeys 48 h after ocular challenge with the hsp60 antigen. The frequency of chlamydia-specific IgG-secreting B cells in conjunctiva varied from 5 to 57 SFC per 10^6 cells; monkeys 17 and 20 exhibited the lowest chlamydia-specific responses. Samples were taken from these two monkeys at day 42 postinfection, when the peak of inflammation would have passed. Cholera toxin-specific IgG-secreting B cells were also detected in conjunctivae, since monkeys had been coimmunized with this antigen before the primary infection (Tables 1 and 2).

Regional draining CLN were also obtained from ocularly immune monkeys on day 14 or 21 after secondary ocular infection. The frequencies of chlamydia-specific B cells in CLN from six monkeys during secondary ocular infection and four naive animals are shown in Table 2. The frequency of chlamydia-specific IgG-secreting B cells for three time points before and after secondary challenge ranged from 24 to 996 SFC per 10^6 cells. No apparent correlation was observed between the number of chlamydia-specific SFC and the time after secondary challenge, since the frequencies of SFC 140 days after primary infection equaled or surpassed those observed 4 weeks after secondary infection. The variability among animals is believed to relate to their individual variations in ocular responses and concomitant differences in follicle formation.

CLN cells obtained on day 14 or 21 after the secondary challenge were also tested against cholera toxin and the negative control antigen, thyroglobulin. As expected, high frequencies of cholera toxin-specific SFC were observed among B cells obtained from cholera toxin-immunized animals. The mean frequency of SFC ranged from 188 to $1,121/10^6$ CLN cells. Vigorous serum antibody responses to cholera toxin were elicited by preimmunization with either conjugated cholera toxin or cholera toxin alone. Very few thyroglobulin-specific SFC were detected at any time (0.0 to 7 SFC per 10^6 cells) and represented nonspecific background, since cells tested on uncoated wells developed similar low numbers of spots (<10 SFC per 10^6 cells). Few SFC were detected for any of the three antigens when normal monkey lymph node cells were tested, further demonstrating the specificity of responses after infection (Table 2). No conjunctival lymphocytes were obtained from clinically normal or naive animals; only rare $CD8^+$ lymphocytes and generally no follicles were present.

Only low numbers of chlamydia-specific or cholera toxin-specific SFC were detected in PBMC from infected monkeys (0 to 30 and 3 to 49 SFC per 10^6 cells, respectively) (Table 2). These lower numbers in peripheral blood parallel our observations with T cells (6). hsp60 appeared to induce a 10- to 20-fold-higher number of IgG-secreting SFC (14 to 57 SFC

TABLE 1. Frequency of chlamydia-specific IgG-secreting B cells in conjunctiva and CLN in infected or ocularly immune monkeys after hsp60 challenge[a]

Monkey no.	Antigen	No. of antigen-specific SFC/10^6 cells			
		Chlamydia specific		Cholera toxin specific	
		CLN	Conj	CLN	Conj
D5	hsp60	57	17	42	8
D6	hsp60	14	2	72	2
329	Live chlamydial EB	43	58	150	260
563	Live chlamydial EB	43	8	20	5
17	Live chlamydial EB	9	465	20	337
20	Live chlamydial EB	5	4	0	0

[a] Conjunctival (Conj) and CLN cells were obtained from actively infected monkeys (monkeys 329 and 563, day 21 postinfection; monkeys 17 and 20, day 42 postinfection) or 48 h after immune monkeys (D5 and D6) received an hsp60 challenge. The numbers of antigen-specific cells were determined by ELISPOT assay as described in the text.

TABLE 2. Mean frequency of chlamydia-specific and cholera toxin-specific IgG-secreting B cells in CLN and PBMC after secondary infection[a]

Group	Monkey no.	Day postinfection	Mean no. of antigen-specific SFC/10^6 cells with the following antigen:					
			Chlamydia		Cholera toxin		Thyroglobulin	
			CLN	PBMC	CLN	PBMC	CLN	PBMC
Secondary infection	D5	0	996	0	2,173	75	0	0
	D8	0	44	3	68	22	0	2
	D7	14	24	6	67	6	2	0
	1955	14	57	6	308	3	7	1
	D6	21	78	27	124	20	0	0.5
	D9	21	227	23	649	14	6	7
Naive controls	C51		1.5	1	0.5	0	1.5	0
	D30		5.5	2	0.5	0	0	0
	D32		2	8	2	4	1.5	0
	D27		6.5	0	8	0	3	0

[a] Lymphocytes were obtained from CLN or peripheral blood at 20 weeks after primary infection before secondary challenge (day 0) and 14 and 21 days later. Tissues were collected from two different monkeys at each time point for ELISPOT assay. CLN cells and PBMC were tested against chlamydial EB, cholera toxin, or thyroglobulin, and SFC in duplicate wells were enumerated.

per 10^6 cells) than of IgA-secreting SFC (0 to 25 SFC per 10^6 cells) in conjunctivae of immune animals. Levels of cholera toxin-specific SFC were similar to those seen for chlamydial antigen for three of four animals (data not shown). In two monkeys, the frequencies of antichlamydial SFC were compared for conjunctiva, regional draining CLN, and distant inguinal lymph nodes. Only background levels of antigen-specific SFC were detected in nondraining distant lymph nodes (0 to 5 SFC per 10^6 cells) and presumably reflect the persistence of specific SFC only locally in conjunctivae and lymph nodes in the region of the infection.

Using the ELISPOT technique, we determined the frequencies of IgG-secreting chlamydia-specific B cells in the conjunctivae, lymph nodes, and peripheral blood during the course of ocular chlamydial infection. Conjunctival follicles, a hallmark of trachoma, are germinal centers predominantly composed of B cells (12, 21). Follicle formation and associated perifollicular T-cell infiltration of chlamydia-infected conjunctivae have been well documented in both experimental and clinical chlamydial eye infections (2, 3, 9, 10, 12). Recently, workers in our laboratories demonstrated by in vitro functional assays that chlamydia-specific lymphocytes are present at the site of ocular infection (20). We showed chlamydia-specific proliferative responses with lymphocytes isolated from conjunctivae of monkeys either during active chlamydial infection or after topical challenge of immune monkeys with noninfectious chlamydial hsp60 antigen. Although at that time we demonstrated in vitro chlamydia-specific antibody secretion by conjunctival B cells, we were unable to quantify the chlamydia-specific B cells at the site of infection (20).

The detection of cholera toxin-specific B cells in conjunctivae is consistent with our previous immunohistochemical studies on the migration of cholera toxin-specific B cells into infected conjunctivae after oral immunization with cholera toxin (15). In most monkeys, the frequencies of chlamydia- and cholera toxin-specific B cells were lower than the frequencies of antigen-specific T cells detected by limiting dilution (6). The hsp60 (and probably other proteins) obtained by Triton X-100 extraction of EB induces an ocular inflammatory response similar to delayed hypersensitivity; the response involves predominantly T cells (20). Follicles reappear in many hsp60-challenged eyes but are more variable than the T-cell infiltrate associated with the inflammatory response. The calculations of total SFC per 10^6 cells are based on a mixture of T and B lymphocytes. Since follicles always appear during active ocular chlamydial infection, we would expect the majority, if not all, of those B cells to be chlamydia specific. The limited availability of conjunctival tissue has made more precise data difficult to obtain.

In all cases, higher frequencies of chlamydia-specific B cells were detected in conjunctivae and regional draining lymph nodes than in PBMC or distant lymph nodes collected at the same times from any monkey. These results are consistent with our previous findings that chlamydia-specific antibody secretion by PBMC in vitro was undetectable even when 10-fold-higher numbers of PBMC were cultured (20). The low or negative ELISPOT results with PBMC are probably due to the fact that PBMC include only about 10% B lymphocytes, compared with approximately 50% B cells in lymph nodes and infected conjunctivae (20). In other studies, we detected substantial numbers of chlamydia-specific T cells in blood (30 to 100/10^6 cells), although these were also at lower levels than those in conjunctivae (6, 20).

Our present findings are important because they better quantify the chlamydia-specific cellular component of the conjunctival inflammatory B-cell response. They indicate that studies of the B-cell responses at distant sites, including the peripheral blood, do not necessarily reflect the extent of the local immune responses in trachoma. Further studies are needed to determine the relative importance of these chlamydia-specific B cells in protective versus pathogenic responses to ocular infection. Studies are planned to further define the persistence of chlamydia-specific B cells in the follicles and regional lymph nodes and their interaction with the large numbers of chlamydia-specific T cells found at these sites.

These studies were supported by a postdoctoral research fellowship from the Fight For Sight Research Division of the National Society to Prevent Blindness (to S.P.), a grant from the Edna McConnell Clark Foundation (to J.A.W.-H.), and Public Health Service grants EY03324 (to J.A.W.-H. and H.R.T.) and RR00130 and RR07002 (to R.B.H.) from the National Institutes of Health.

We thank Shirley Johnson, Vivian Velez, and Lori DeJong for their technical assistance in these studies. We particularly thank E. Talor for help in adapting the ELISPOT protocol to chlamydiae.

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