Long-Term Clinical, Microbiological, and Immunological Observations of a Volunteer Repeatedly Infected with *Chlamydia trachomatis*

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A blind volunteer was inoculated in one eye with an isolate of *Chlamydia trachomatis* in 1961 and followed for 20 years. During this time, many observations were made of his clinical responses to the first inoculation and several subsequent inoculations with the same and other strains, chlamydial shedding, and antibody and cell-mediated immune responses. Evidence is presented that partial resistance to chlamydial eye infection developed during repeated infections and that antibodies, cell-mediated immune reactions, and specific antigen in conjunctival cells persisted for many years after the last infection. The antibody response was directed mainly against the original infecting immunotype, regardless of whether the response was restimulated by infection with the same immunotype or with other immunotypes. The lymphocyte stimulation response appeared to be species specific.

Interest in chlamydial infections has increased in recent years, reflecting the current high incidence of sexually transmitted diseases caused by chlamydiae. However, it has rarely been possible to follow infected individuals over a long period and to evaluate their immune responses to such infections.

We have had the unique opportunity to record clinical, microbiological, and immunological findings for one volunteer over 20 years. The infection was ocular, permitting direct evaluation of clinical disease.

MATERIALS AND METHODS

Volunteer. In 1961, a single, white, near-blind male, 45 years of age, volunteered as a subject and gave fully informed consent (Committee for Human Research protocol no. 280201) to our studies. The conjunctivas, corneas, and limbes of both eyes were normal when examined with a binocular loupe and a slit lamp. In the posterior segments, both eyes showed the changes of advanced retinitis pigmentosa. Vision was reduced to the ability to see hand movements.

Inocula. Between March 1961 and October 1967, three strains of *Chlamydia trachomatis*, all isolated in this laboratory, were used for the experimental infections (Table 1).

A 0.02-ml volume of the inoculum, diluted as indicated below, was placed on the everted upper lid of the right eye and gently spread with a glass rod.

Examinations. Both eyes were examined at intervals of days or weeks, depending on clinical activity. Conjunctival scrapings were collected for Giemsa

staining and, beginning in 1963, also for immunofluorescence staining. Specimens for reisolation of the chlamydiae from the yolk sacs of embryonated chicken eggs were collected from the time of each inoculation until two consecutive negative cultures had been obtained. Serum was obtained at the time of most examinations, and tears were occasionally collected, as previously described (6).

Clinical examinations. Both eyes were examined with a binocular loupe and a slit lamp for evidence of purulent discharge, conjunctival hyperemia, follicular or papillary hypertrophy, limbal edema, neovascularization, and micropannus development.

Reisolation of chlamydiae. Conjunctival scrapings suspended in broth-saline were injected into the yolk sacs of embryonated chicken eggs, as previously described (8). Three blind passages were made before a specimen was considered negative. Death of the embryo in the absence of bacterial contamination, together with microscopic demonstration of elementary bodies in Giemsa-stained yolk sac smears, denoted a positive specimen.

Microscopic examination of conjunctival scrapings. Standard methods of Giemsa staining and microscopic examination of conjunctival scrapings were followed. From 1963 onward, conjunctival scrapings were also examined by direct immunofluorescence (9) with sera from rabbits immunized against immunotypes E, F, and L2. The scrapings were placed within etched circles approximately 1 cm in diameter, and the entire area was examined with a $\times 40$ oil immersion objective.

Immunological tests. Complement fixation tests were performed in the laboratory of the late K. F. Meyer, Hooper Foundation, San Francisco, Calif., with a standard boiled chlamydial group-reactive anti-

TABLE 1. *C. trachomatis* strains used for infection

Strain	Immuno-type	Source of isolate
IC-Cal-3 (yolk sac grown)	F	Inclusion conjunctivitis, newborn (age, 15 days), San Francisco, 1960 (3)
IC-Cal-8 (yolk sac grown and cell culture grown)	D	Inclusion conjunctivitis, newborn (age, 11 days), San Francisco, 1961 (11)
AP-2 (yolk sac grown)	B _a	Trachoma, early active, child (age, 7 yr), Arizona, 1960 (3)

gen (*C. psittacosis* 6BC) on sera collected from March to July 1961. Complement fixation tests were also performed in our laboratory by a comparable procedure but with boiled group-reactive *C. trachomatis* AP-2 antigen (unpublished data) on sera collected from March 1961 to April 1964. All sera and selected tears were tested by a modification of the microimmunofluorescence technique of Wang, with nine immunotype-specific antigens (7).

Cross-absorption studies on selected serum samples. Samples of seven sera were absorbed individually with one of nine antigens of *C. trachomatis* immunotypes C, A, F, B, B_a, D, E, L1, and L2 or with one antigen of *C. psittaci* 6BC, as previously described (1). The absorbed sera were then titrated by microimmunofluorescence as described above.

Cell-mediated immune response. On multiple occasions between January 1976 and September 1980, lymphocyte stimulation (10) and leukocyte migration inhibition were measured (8a). Three chlamydial antigens were used: *C. trachomatis* immunotypes L2 (strain LB-1) and F (strain IC-Cal-3) and *C. psittaci* 6BC.

RESULTS

Observations during period of experimental infections (1961 to 1968). The first inoculation of this volunteer with chlamydiae has been previously reported in detail (15). Approximately $10^{2.6}$ 50% egg infective doses (EID₅₀) of IC-Cal-3 (immunotype F) were placed onto the conjunctiva. Previous work (3) had suggested that 10 EID₅₀ constituted one infective dose for the human adult eye. A summary of clinical and laboratory observations made during the initial infection is shown in Table 2. The first evidence of infection was noted on day 7 and consisted of follicular and papillary hypertrophy. Giemsa-stained inclusions were seen at that time, and chlamydiae were reisolated. Serum antibodies demonstrable by microimmunofluorescence appeared 3 weeks after inoculation. Serum antibodies demonstrable by complement fixation tests appeared at the same time but never ex-

TABLE 2. Summary of initial experimental inoculation of infectious chlamydiae onto the conjunctivas of a human volunteer^a

Day	Date (1961)	Evidence of infection ^b			Serum antibody titer (MIF) against semipurified antigen ^c					
		Agent reisolated	Inclusions observed ^d	Clinical activity	C	A	B	DE	F	6BC
0	3/14	ND	—	—	—	—	—	—	—	—
7	3/21	+	—	+	—	—	—	—	—	—
14	3/28	+	+	+	—	—	—	—	—	—
22	4/4	+	+	+	10	10	10	10	80	—
29	4/11	+	+	+	20	40	40	40	160	—
43	4/25	+	+	+	10	20	20	40	160	—
58	5/11 ^e	+	+	+	20	20	20	10	160	—
69	5/22	—	—	±	10	10	10	—	80	—
84	6/6	—	—	—	—	—	—	—	80	—
94	6/16	ND	—	—	—	—	—	—	80	—
118	7/10	ND	—	—	—	—	—	—	20	—
206	10/6	ND	—	—	—	—	—	—	20	—

^a Strain IC-Cal-3 (immunotype F) at $10^{2.3}$ EID₅₀ was used for the inoculum on day 0.

^b +, Yes; —, no; ±, marginal evidence; ND, not done.

^c Numbers are the reciprocals of the serum dilutions resulting in positive immunofluorescence; —, nonreactive in dilution of 1:10; MIF, microimmunofluorescence.

^d Giemsa stain only.

^e Antimicrobial therapy begun.

TABLE 3. Summary of 11 consecutive experimental inoculations of infectious chlamydiae onto the conjunctivas of a volunteer

Inoculation no.	Date of inoculation	Inoculum			Days after infection ^a		Inclusions observed ^b	
		Strain	Immuno-type	Approx EID ₅₀	Clinical activity	Agent reisolated	Giemsa	FA
1	3-14-1961	IC-Cal-3	F	10 ^{2.3}	7-58	6-58	+	
2	4-9-1962	IC-Cal-3	F	10 ^{3.3}	14-35	24-29	+	
3	7-26-1962	IC-Cal-3	F	10 ^{3.3}	11-28	25	+	
4	1-25-1963	IC-Cal-3	F	10 ^{5.0}	3-5	3-20	+	
5	4-24-1963	IC-Cal-3	F	10 ^{5.0}	6-15	2	+	
6	6-11-1963	IC-Cal-3	F	10 ^{5.0}	Negative	6-14	+	
7	10-8-1963	AP-2 ^c	B _a	10 ^{5.3}	2-44	2-27	+	+++
8	4-7-1964	IC-Cal-3	F	10 ^{2.3}	Negative	Negative	-	-
9	11-2-1964	IC-Cal-8 ^d	D	10 ^{2.0}	14-59	ND ^e	+	+++
10	5-25-1965	IC-Cal-8	D	10 ^{2.0}	Negative	Negative	-	+
11	10-4-1966	IC-Cal-8	D	10 ^{6.0}	12	9-10	+	+

^a Antimicrobial agents were administered beginning at day 58.

^b FA, Fluorescent antibody; +, some inclusions observed; + + +, many inclusions observed; -, no inclusions observed.

^c Left eye was inoculated (see text).

^d Cell culture grown (see text).

^e ND, Not done.

ceeded a titer of 1:20 and disappeared early. Beginning on day 58 and continuing for 2 weeks, the volunteer received 0.5 g of sulfamethoxypyridazine daily by mouth and drops of 1% tetracycline hydrochloride in oil three times daily in the eye. After the therapy was begun, chlamydiae could not be reisolated, no Giemsa-stained inclusions were seen, and the clinical activity subsided.

Between 1961 and 1967, the eyes of the volunteer were repeatedly challenged with infective inocula, heat-inactivated chlamydial materials, or normal yolk sac. The latter two materials were used to test for hypersensitivity of the conjunctivas. They elicited no reactions and are not discussed here any further.

Eleven inoculations with infective material are summarized in Table 3. When the data for inoculations 2 and 3 are compared with those for inoculation 1, there appears to have been a delay in the onset of clinical activity after the later inoculations, with fewer reisolations of chlamydiae. However, the delay was apparently overcome by the subsequent use of a 50-fold-larger infective dose in inoculations 4 and 5. The development of partial host resistance is suggested by the shortened periods of clinical and microbiological activities after inoculations 3 through 6. Antimicrobial drugs were given only after inoculation 1 and not after any later inoculations.

Two and one-half years after the first inoculation into the right eye, another strain, AP-2 (immunotype B_a), isolated from a child with typical endemic trachoma, was inoculated into

the left eye. Previous experience had indicated that much more strain AP-2 than genital strain IC-Cal-3 is required to produce infection in eyes of monkeys (2). Infection was apparent clinically and by laboratory tests within 48 h. The fluorescent antibody-stained conjunctival scrapings were more consistently inclusion positive than were Giemsa-stained smears and had larger numbers of inclusions. No chlamydiae could be recovered 4 weeks after inoculation in the absence of chemotherapy, and the eye was clinically normal after an additional 2 weeks.

In April 1964, 3 years after the initial infection, IC-Cal-3 was again inoculated into the right eye at the same concentration as that of inoculum 1. However, there was no clinical or laboratory evidence of infection; there appeared to be complete resistance to this infective dose.

In November 1964, another inclusion conjunctivitis strain, IC-Cal-8 (immunotype D), which had been maintained in a cell culture for seven passages (4), was inoculated into the right eye in a dose of approximately 10² EID₅₀. The incubation period was longer (14 days), and the onset of conjunctivitis was insidious, but inclusions were regularly demonstrable by immunofluorescence from day 2 onward and intermittently demonstrable by Giemsa staining from day 7 onward for at least 2 months. No attempt was made to reisolate the chlamydiae in yolk sacs.

The last two inoculations were with yolk sac-grown IC-Cal-8 (immunotype D). A small inoculum, 10³ EID₅₀, administered 6 months after the first exposure to this strain, did not result in infection; 6 months later a larger inoculum, 10⁶

TABLE 4. Chlamydial inclusions in conjunctival scrapings as demonstrated by direct immunofluorescence^a

Time period	No. of infections	Inclusions (no. positive/total specimens) ^b
1961 to 62	3	ND
1963 to 64	6	46/57 (81)
1965 to 68	2	67/172 (39)
1969 to 71	0	18/52 (35)
1972 to 74	0	8/42 (19)
1975 to 77	0	8/78 (10)
1978 to 80	0	3/36 (8)

^a Scrapings were from either or both eyes.^b Numbers in parentheses indicate percent positive. ND, Not done.

EID₅₀, resulted in a clinical infection which was confirmed by reisolation of chlamydiae and by microscopically evident inclusions.

The results of microscopic examination of fluorescent antibody-stained conjunctival scrapings are shown in Table 4. During the period of frequent experimental infections, inclusions were regularly seen, paralleling the intensity of disease. During subsequent years of observation, in the absence of any overt infection, the proportion of inclusion-positive specimens gradually declined. In those specimens that were inclusion positive, only one or two inclusions could be located. They were found in scrapings from either or both conjunctivas. Such inclusions gave evidence for the long persistence of antigen, although viable chlamydiae had last been recovered in 1966.

Observations of clinical disease. After each of the first two inoculations with IC-Cal-3 (1961 and 1962), the inoculated eye developed follicular conjunctivitis with hyperemia, discharge, epithelial keratitis, and preauricular lymphadenopathy. The first episode was treated as described above. The second episode subsided spontaneously about 5 weeks after inoculation. Subsequent inoculations with this strain produced only transient conjunctivitis, which was relatively mild and subsided spontaneously in 5 to 17 days.

During subsequent inoculation with strain AP-2 (immunotype B_a), isolated from a patient with classic trachoma, the volunteer developed an extension of superficial blood vessels at the superior limbus (micropannus). During the inoculations of the other eye with immunotypes F and D, a similar vessel extension developed in the inoculated eye, but to a lesser extent. In both eyes, this vessel extension was minimal and just detectable with a slit lamp.

After the final inoculation (1967), the volunteer was examined at frequent intervals. Between 1968 and 1981 he did not have an overt

bout of conjunctivitis. A few lymphoid follicles, considered to be within the range of normal, were noted in the conjunctivas. The corneas remained clear, with no scarring. Thus, the clinical appearance of the external eye remained normal throughout this period (1967 to 1981).

Observations on antibody responses (1961 to 1980). Tears for antibody determinations were collected beginning in 1972, 6 years after the last experimental inoculation. No antibodies were demonstrated in tears from the left eye, which was infected (with strain AP-2) only one time. Only low titers of antibodies, mainly reactive with IC-Cal-3, were present in tears from the right eye.

Sera were collected before the initial inoculation and at intervals throughout the period of observation. During and after the first experimental infections with immunotype F, only immunotype F antibody levels were persistently elevated. After infection with immunotype B_a, levels of antibodies to the BDE complex rose but never reached the level of the immunotype F antibodies. In the years after the last infection, the higher titer of immunotype F antibodies persisted, as did the lower titers of antibodies to other immunotypes.

In an attempt to determine specificities, we absorbed out antibodies with representative immunotype antigens, including the three used for inoculation, F, B_a and D (Table 5). Unabsorbed serum 6, collected 4 weeks after the first inoculation and 3 weeks after the onset of disease, showed a titer of 1:160 against immunotype F but showed a titer of only 1:20 against the other immunotypes. Absorption with any of the heterologous antigens removed all species-specific activity, leaving antibody which reacted only with and which could be absorbed only by immunotype F antigen.

Serum 47, collected 2 years later, after repeated infection of one eye with immunotype F and one infection of the other eye with immunotype B_a, had similar titers against all of the antigens tested. But again, immunotype antigens A, B, B_a, C, D, and E removed the species-specific antibodies, whereas only antigen F removed anti-immunotype F antibody.

Ten years later (1973), after additional infections with immunotypes F and D, antibody titers had changed little. Twelve and one-half years after the first infection with immunotype F and almost 6 years after the last infection with immunotype D, the principal antibody was still directed toward immunotype F. Table 5 shows the results of more comprehensive absorptions of this serum. Again, only immunotype F antigen could remove the specific antibody. Two strains of lymphogranuloma venereum were no more effective than were the other heterologous

TABLE 5. Reactivity of sera after absorption with different immunotypes of chlamydiae

Serum date	Inoculation history ^a	Absorbing antigen	MIF titer against ^b :									
			A	B	C	D	E	F	B _a	L1	L2	6BC
1961	IC-Cal-3 (F) (1 time)		20	20	20	20	20	160	20			
		A, B, B _a , C, D, or E	—	—	—	—	—	160	—			
		F	—	—	—	—	—	—	—			
1963	IC-Cal-3 (F) (6 times)		160	160	160	160	160	160	160			
		AP-2 (B _a) (1 time)	—	—	—	—	—	160	—			
		F	—	—	—	—	—	—	—			
1973	IC-Cal-3 (F) (7 times)		40	40	40	40	40	160	40	20	20	—
		AP-2 (B _a) (2 times)	—	—	—	—	—	80	—	—	—	—
		IC-Cal-8 (D) (3 times)	—	—	—	—	—	—	—	—	—	—
		6BC	40	40	40	40	40	160	40	40	40	—

^a Immunotypes are indicated in parentheses.

^b Numbers are the reciprocals of the serum dilutions resulting in positive immunofluorescence; —, nonreactive in a dilution of 1:10; MIF, microimmunofluorescence.

antigens. *C. psittaci* 6BC showed no cross-reactivity and was unable to absorb out any of the antibody activity.

From 1976 to 1980, we observed the cell-mediated immune responses of the volunteer. Both lymphocyte stimulation and leukocyte migration were somewhat elevated (8a, 10). Although stimulation by *C. psittaci* 6BC antigen was minimal, the levels of stimulation obtained with antigens IC-Cal-3 (immunotype F) and LB-1 (immunotype L2) were comparable (unpublished data).

DISCUSSION

The dose of the infective agent determined the establishment of infection. The timing of the various inoculations did not permit any evaluation of levels of resistance. However, it is tempting to postulate that inoculation 5 induced resistance to an infective dose of the homologous strain administered 6 weeks later. The gradually increased resistance of the volunteer was suggested by the following. (i) Increasingly larger doses of infective material were necessary for *C. trachomatis* to produce infection. (ii) The incubation period lengthened for repeated inoculations with a given strain. (iii) The duration of the disease process shortened. (iv) The intensity of reaction decreased.

Reisolation of chlamydiae from the conjuncti-

vas indicated the specific etiology of clinical signs. However, reisolation in chicken embryo yolk sacs is cumbersome and time-consuming. Present-day cell culture methods would have been valuable but were not available in the earlier part of the study, when numerous inclusions were visible in conjunctival smears. In later years, when cell culture methods became available and immunotypes could be determined, isolations were not attempted because of the scarcity of inclusions, a feature usually incompatible with isolation of chlamydiae in our experience. Microscopic examination of conjunctival scrapings by staining with Giemsa or later by immunofluorescence was rapid and reliable throughout. We have stressed in the past that immunofluorescence was consistently more sensitive than Giemsa staining (9). Inclusions were demonstrated by immunofluorescence during the incubation period and at a time when the disease was subsiding. Also, greater numbers of inclusions were identified by immunofluorescence than were identified in Giemsa-stained preparations. As mentioned above, inclusions seen by immunofluorescence indicated the presence of antigen but not necessarily that of viable chlamydiae, supporting the belief that long latency is a feature of chlamydial eye infection in the absence of any clinical activity (5, 12).

The acute eye disease which resulted from repeated inoculation of chlamydiae resembled

that resulting from natural infection. However, the acute inflammatory changes always subsided and, in the absence of bacterial infection, did not lead to chronic progressive changes. The limited extension of superficial blood vessels in the cornea resembled the micropannus observed by Mordhorst et al. in children after protracted eye infection with genital chlamydiae (14).

Beginning with the initial experimental infection and continuing throughout the study, infection with any of the three immunotypes stimulated increased production of circulating antibodies. At all times, the response to the initial inoculum, IC-Cal-3 (immunotype F), was greater than those to other immunotypes, including immunotypes B_a and D, which were used for later inoculations. The specificity of this antibody response was confirmed by multiple absorption studies in which only immunotype F antigen was capable of removing all measurable antibody activity from serum samples, even after repeated inoculation with other immunotypes.

Procedures for evaluation of cell-mediated immune responses were not available to us until 10 years after the last inoculation (8a, 10). No information is available on the specificity of the cell-mediated immune responses to the initial infection, i.e., whether they were type specific or species specific, as appears at the present time.

The serendipitous choice of IC-Cal-3 (immunotype F) for the initial inoculation permitted an evaluation of serum antibody response which would not have been possible with comparable strains available at the time, since immunotype F is antigenically distinct from and does not overlap with other immunotypes isolated in our area.

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