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IMMUNITY TO REINFECTION OF THE GENITAL TRACT OF MARMOSETS WITH *CHLAMYDIA TRACHOMATIS*

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Summary.—Eleven marmosets inoculated intra-vaginally with either of 2 serotypes (D/E and H) of *Chlamydia trachomatis* developed a self-limited infection which persisted usually for 10–42 days. Animals re-inoculated on one or more occasions were, however, infected generally for a shorter duration, usually 3–7 days. Curtailed infections were observed after re-inoculation with either the same or a different serotype, indicating that immunity was not serotype specific but cross-protective. IgM and/or IgG chlamydial antibody, measured by micro-immunofluorescence, developed in most of the marmosets on primary infection and was not serotype specific. The antibody titres were boosted on re-infection and there was a correlation between pre-existing high antibody titres and infections of short duration. Chlamydial infection of the genital tract was accompanied by acute inflammation which persisted in about half of the immune animals for up to several weeks despite rapid clearance of the organisms. These features of the experimental infection should help to provide a greater understanding of the immunobiology and pathogenesis of chlamydial genital-tract infections of humans.

CHLAMYDIA TRACHOMATIS is an important cause of a variety of ocular and genital infections in man (Taylor-Robinson and Thomas, 1980). In recent years, several groups of workers have reported that various species of non-human primate are susceptible to experimental chlamydial infection of the genital tract, and that such models are useful for studying the pathogenesis of chlamydial disease (Digiacomo *et al.*, 1975; Jacobs, Arum and Kraus, 1978; Johnson *et al.*, 1980; Møller and Mårdh, 1980; Ripa *et al.*, 1979). However, with the exception of one study in which 2 baboons were used (Digiacomo *et al.*, 1975), there have not been any reports on the immunity or otherwise of primates to re-infection of the genital tract with *C. trachomatis*. In the present study, we describe the results of attempts to re-infect the genital tract of female marmosets with either the same chlamydial strain or another strain of a different serotype.

MATERIALS AND METHODS

Marmosets.—Female marmosets (*Callithrix jacchus*), bred at the Clinical Research Centre, were used. The care and feeding of the animals has been described previously (Furr, Taylor-Robinson and Hetherington, 1976).

Chlamydia trachomatis.—Two strains of *C. trachomatis* were used. Strain 78α, previously (Johnson *et al.*, 1980) incorrectly referred to as serotype F/G, belonged to serotype D/E as determined by a one-way micro-immunofluorescence test (Wang, Kuo and Grayston, 1973). Strain UW4, originally isolated at the University of Washington, Seattle, was obtained from the Institute of Ophthalmology, London, and was typed as serotype H. Organisms were cultured

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in cycloheximide-treated McCoy cells (Evans and Taylor-Robinson, 1979) and stored in sucrose–phosphate (2SP) medium (Gordon *et al.*, 1969) in liquid nitrogen. The same stock pool of each strain was used throughout the study.

Experimental design.—The design of and time interval between experiments was determined primarily by the numbers of animals available. In every experiment, at least one animal that had not been inoculated previously with chlamydiae was included to check the infectivity and effect of the inoculum.

Inoculation of marmosets.—Chlamydial samples were thawed at 37° and agitated on a vortex mixer. They were diluted as required with Eagle's minimal essential medium which contained 10% (v/v) heat-inactivated (56°, 30 min) foetal calf serum, 0·5% (w/v) glucose, vancomycin (100 μg/ml) and streptomycin (50 μg/ml), the pH being adjusted to 7·0 with 4·4mM bicarbonate. A 0·3 ml volume of the chlamydial suspension was then inoculated into the vagina of each animal using a sterile catheter. An anaesthetic was not used as the animals were easily restrained manually.

As sufficient animals were not available at any one time to titrate the infectivity of each stock pool of *C. trachomatis*, the dilution of the pool of strain 78α that previously had been shown reproducibly to infect marmosets (Johnson *et al.*, 1980) was used routinely. An arbitrarily chosen dilution of the pool of Strain UW4 was tested (Expt. No. 4, Table I) and, as it was found to infect, a similar dilution was used thereafter. After inoculation, the residual inoculum was re-frozen and its viability checked subsequently.

Isolation of chlamydiae.—After inoculation, the lower genital tract of each animal was sampled repeatedly at intervals of 3 or 4 days. On each occasion, a nasopharyngeal calcium alginate swab (Wilson Diagnostics Inc., Illinois, U.S.A.) was inserted into the vagina and then expressed in 1 ml of cold 2SP medium containing 10% (v/v) heat-inactivated foetal calf serum. The samples were agitated on a vortex mixer, and 0·5ml volumes were dispensed into sterile ampoules containing glass beads and stored in liquid nitrogen until they were tested. To detect the presence of chlamydiae, the content of each vial was thawed and inoculated on to a pair of McCoy cell monolayers on 13mm coverslips in flat-bottom tubes. The inoculated cell cultures were centrifuged at 2,800 *g* for 1 h and after incubation at 37° for a further 2 h the medium was removed and replaced with medium which contained cycloheximide (2 μg/ml). The cultures were incubated at 37° for 48–72 h, after which the cells were fixed with methanol, stained with Giemsa, and examined for the presence of chlamydial inclusions by dark-ground microscopy. All cell monolayers were coded before examination to avoid subjective bias when seeking inclusions.

Cytological examination.—After taking a swab for the isolation of chlamydiae, a second vaginal swab was smeared on a microscope slide. Smears were fixed in methanol, stained with Giemsa and coded before being examined microscopically.

Serological studies.—Antibody to a range of *C. trachomatis* serotypes was measured in marmoset sera by means of a micro-immunofluorescence technique (Thomas, Reeve and Oriel, 1976). Sheep anti-human IgM and sheep anti-human IgG antibody, both conjugated with fluorescein isothiocyanate (Wellcome Reagents Ltd), were used.

Administration of cyclophosphamide.—Four animals infected previously with chlamydiae, but from which the organisms could no longer be isolated on repeated attempts, were given cyclophosphamide in an effort to re-activate a possible latent infection. The animals were not weighed immediately before receiving cyclophosphamide, but on the basis of previous weight recordings were estimated to weigh 250–300 g. Each animal was injected intramuscularly with 1 ml (0·5 ml in each leg) of a solution of cyclophosphamide (25 mg/ml) (Endoxana, WP Pharmaceuticals Ltd) reconstituted immediately before use, thus providing a dose of approximately 83–100 mg/kg body wt.

Statistics.—The correlation between the titre of serum antibody before inoculation and the duration of infection was determined by means of an exact test of a 2×2 table using 2 medians (Hill, 1979).

RESULTS

A series of 5 experiments involving 11 marmosets (designated A to K) was performed, in which groups of animals were re-inoculated at various intervals with either the same or a different strain of *C. trachomatis*, and the duration of each infection recorded. Titration of inocula in McCoy cells showed that marmosets inoculated with strain 78α received 4·2–$8{\cdot}8 \times 10^5$ inclusion-forming units (i.f.u.), and animals inoculated with strain UW4 received $3{\cdot}2$–$5{\cdot}9 \times 10^4$ i.f.u.

Duration of the first infection

Eight animals (A–H) were inoculated for the first time with strain 78α. The duration of infection in all of these animals but one was within the range 10–42 days, the mean duration being 26 days. The duration of infection in 3 animals (I, J, K)

TABLE I.—*Duration of chlamydial infection as assessed by isolation of viable organisms from marmosets inoculated intravaginally with* C. trachomatis

Experiment number		Duration of infection (days) in indicated animal										
		A	B	C	D	E	F	G	H	I	J	K
1		10[b]	3	42	39							
	↓89[a]											
2		30	49	13	13	13						
	↓55											
3		3	7	7	3		38	38				
	↓64											
4		3†	6	3†	16		3	<3†	21	43†		
	↓94											
5		3†	3†		18†		3†	3†	10†	10†	31†	21†

[a] ↓n n = interval in days between the start of successive experiments.
[b] Animals were inoculated with strain 78α unless marked †, which indicates inoculation with strain UW4.

inoculated initially with strain UW4 ranged between 21 and 43 days, the mean being 32 days.

Duration of infection after the second inoculation

Eight animals were inoculated for a second time with either the homologous or heterologous strain. Four of them (A–D) inoculated initially with strain 78α were re-inoculated with the same strain 89 days later (Expt. No. 2, Table I). The mean duration of infection was 26 days (Table II), which is identical to the mean duration of infection in the animals infected with strain 78α for the first time. Furthermore, 2 of the animals (C and D) were infected for 13 days, which was the same duration as that seen in the control marmoset (E) inoculated for the first time in Expt. No. 2.

In contrast, in a subsequent re-challenge experiment (No. 4, Table I), in which the interval between the first and second inoculation of 2 animals (F and G) was 64 days, both exhibited marked immunity. Thus, animal F, inoculated on both occasions with Strain 78α, was infected for 3 days only, while animal G, inoculated originally with strain 78α and re-challenged with strain UW4, was chlamydia-negative, even on Day 3.

In Expt. No. 5 (Tables I and II), in which the interval between the first and second inoculation was 94 days, 2 animals

TABLE II.—*Duration of infection in marmosets inoculated intravaginally with* C. trachomatis *on one or more occasion*

Experiment[a] number	Strain of *C. trachomatis* inoculated	Number of previous infections	Marmosets studied	Duration of infection in days
2	78α	0	E	13
		1	ABCD	30, 49, 13, 13 ($\bar{x}=26$)[b]
3	78α	0	FG	38, 38 ($\bar{x}=38$)
		2	ABCD	3, 7, 7, 3 ($\bar{x}=5$)
4	78α	0	H	21
		1	F	3
		3	BD	6, 16 ($\bar{x}=11$)
4	UW4	0	I	43
		1	G	<3
		3	AC	3, 3 ($\bar{x}=3$)
5	UW4	0	JK	31, 21 ($\bar{x}=26$)
		1	HI	10, 10 ($\bar{x}=10$)
		2	FG	3, 3 ($\bar{x}=3$)
		4	ABD	3, 3, 18 ($\bar{x}=8$)

[a] Refers to Expt. No. shown in Table 1.
[b] Mean duration of infection.

(H and I) inoculated with strain UW4 were both infected for 10 days. Despite infection, these animals were considered to be partially immune because the duration of the infection was shorter than that of 2 control animals (J and K) which had been inoculated for the first time (Table I). This partial immunity to strain UW4 was seen in both animals even though one had been infected previously with strain 78α.

Duration of infection after the third, fourth or fifth inoculation

Three experiments were performed in which the duration of infection in animals that had been inoculated on 2 or more occasions previously was compared with the duration in animals inoculated for the first time (Tables I and II). Four animals (A–D) that had been infected on 2 occasions previously with strain 78α were re-challenged with this strain 55 days after the last inoculation (Expt. No. 3, Table I). The mean duration of infection in these animals was 5 days (Table II), which was significantly shorter ($P < 0{\cdot}001$; Student's t test) than the 38-day period of infection in the 2 control animals (F and G).

Two animals (B and D) inoculated on 3 earlier occasions with strain 78α were re-challenged with the homologous strain 64 days after the previous inoculation (Expt. No. 4, Table I). Animal B was infected for 6 days, indicating some degree of immunity. Animal D appeared to eliminate its infection after day 3, since swabs taken on days 6, 10 and 13 were chlamydia-negative. Chlamydiae were, however, isolated on day 16, although 6 subsequent swabs taken over a further period of 3 weeks were negative.

Two animals (A and C) which had been inoculated on 3 previous occasions with strain 78α were re-challenged with strain UW4 64 days after the third inoculation (Expt. No. 4, Table I). The mean duration of infection with strain UW4 was 3 days, in contrast to the 43-day period of infection in the control (Table II), indicating a marked degree of immunity.

In the final re-challenge experiment, 5 animals (A, B, D, F, G) that had been inoculated on 2 or more earlier occasions were inoculated with strain UW4 94 days after the previous inoculation (Expt. No. 5, Table I). Infection lasted 21–31 days in 2 control animals (J and K) receiving strain UW4 for the first time. In contrast animal G, which had been inoculated previously with both strain 78α and strain UW4, and animal F, which had received strain 78α only, were infected for 3 days only. Animals A and B, that had been inoculated on 4 prior occasions, showed similar immunity to re-challenge with strain UW4, being infected for 3 days only (Table II). As before, however, animal D was not consistently negative for chlamydiae. Thus, chlamydiae were isolated on days 3, 7 and 18, but not on days 10, 14, 21 and 31.

Administration of cyclophosphamide

In two experiments (Nos. 4 and 5), chlamydiae were isolated inconsistently from animal D. This suggested that animals might be infected despite the failure to isolate chlamydiae. In an attempt to exacerbate any undetected latent infection, 4 animals (F, G, J, K) that had apparently eliminated their infection were given cyclophosphamide 76 days after they had been inoculated with strain UW4 (Expt. No. 5). Animals J and K had been inoculated only once with Strain UW4, while animals F and G had been inoculated a total of 3 times (Table I). Animal D was not available for testing. Each animal was swabbed 4 and 6 days after receiving cyclophosphamide, but chlamydiae were not isolated.

Inflammatory response of the genital tract

Examination of Giemsa-stained vaginal smears from uninoculated marmosets revealed epithelial cells only, although smears from animal D contained moderate to large numbers of polymorphonuclear (PMN) leucocytes. After inoculation an acute inflammatory reaction occurred as described previously (Johnson *et al.*, 1980).

Genital-tract smears contained predominantly PMN leucocytes, although occasional mononuclear cells were seen also. Inflammation was seen not only in animals inoculated for the first time, but also in those which had been re-inoculated on one or more subsequent occasions. However, in one experiment (No. 4, Table I), chlamydiae were not isolated from animal G after re-inoculation, and it failed to mount a PMN leucocyte response. Nevertheless, in a subsequent experiment (No. 5, Table I) in which this animal was infected for 3 days, PMN leucocytes were observed in moderate numbers on day 3 but not thereafter.

After the first inoculation of each animal, the PMN leucocyte response persisted as long as chlamydiae were isolated but insufficient swabs were taken to determine whether it persisted appreciably longer. After re-inoculation on two or more occasions, the PMN leucocyte response persisted in about half of the animals for longer than the duration of chlamydial isolation. The excess duration of inflammation relative to isolation was 1–3 weeks.

Serological response

Antibodies to *C. trachomatis* were not detected in sera from 9 of the 11 marmosets before inoculation. Animal B had serum IgG antibody at a titre of 1:2 while animal J had IgM and IgG antibodies, both at a titre of 1:4. After the first inoculation of *C. trachomatis*, IgM and/or IgG antibody was detected in the serum of 10 of the marmosets (Table III). Even after multiple inoculations the highest titre of IgM antibody detected in 9 of the 11 animals was 1:16 or less, the other 2 animals having maximum IgM antibody titres of 1:64. In contrast, the titre of IgG antibody tended to increase with subsequent inoculations (Table III). Five animals developed titres of 1:512 or greater, while 2 other animals developed maximum IgG antibody titres of 1:128.

The antibody in sera from infected marmosets was not specific for the serotype of *C. trachomatis* with which each animal had been inoculated, reacting to the same extent with all the serotypes used as antigens in the micro-immunofluorescence test. This phenomenon was observed with sera from all animals, irrespective of the number of inocula they had received or the strain inoculated.

Relationship between immunity to re-infection and chlamydial antibody in serum

Antibody titres were determined within 5 days before challenge. Animals inoculated with strain UW4 which had IgM antibody at a titre of 1:4 or greater or IgG antibody at a titre of 1:32 or greater before challenge tended to eliminate their infections within 1 week, while animals with IgM or IgG antibodies at lower titres tended to be infected for 10 days or

TABLE III.—*Humoral antibody response in marmosets inoculated on one or more occasion with* C. trachomatis

Inoculation	Class of antibody detected	Maximum antibody titres[a] in sera from indicated animals										
		A	B	C	D	E	F	G	H	I	J	K
Pre-	IgM	<2	<2	<2	<2	<2	<2	<2	<2	<2	4	<2
First	IgM	<2	4	4	4	8	<2	8	<2	2	64	<2
Second	IgM	8	64	8	8		16	8	8	4		
Third	IgM	8	32	8	8		16	16				
Fourth	IgM	16	32	8	4							
Fifth	IgM	16	64		4							
Pre-	IgG	<2	2	<2	<2	<2	<2	<2	<2	<2	4	<2
First	IgG	4	8	8	32	<2	<2	512	2	4	512	16
Second	IgG	64	2048	32	2048		128	128	16	32		
Third	IgG	64	1024	64	256		64	64				
Fourth	IgG	256	2048	128	512							
Fifth	IgG	2048	1024		64							

[a] Reciprocal of highest serum dilution showing fluorescence.

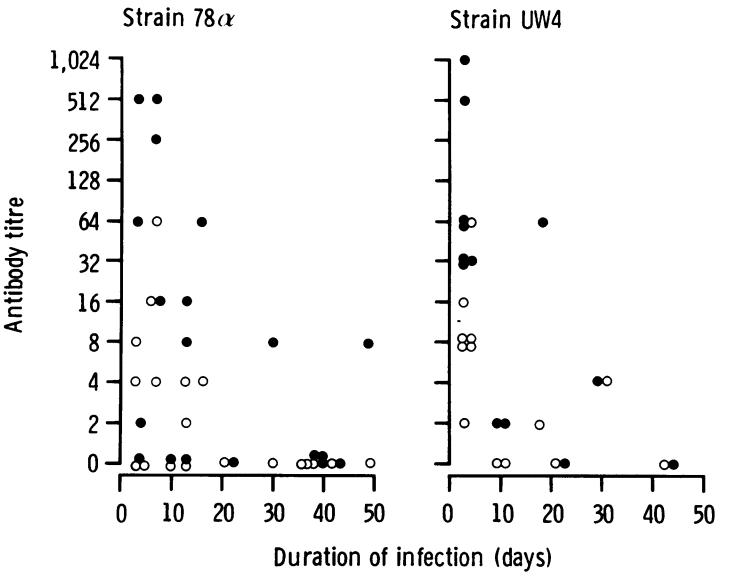

FIG.—Relationship between pre-existing titres of anti-chlamydial antibody in sera and duration of infection in marmosets inoculated with either of 2 strains (78α and UW4) of *Chlamydia trachomatis*. (○ IgM; ● IgG).

longer ($P=0{\cdot}03$) (Fig.). On the other hand, although there appeared to be an association between pre-existing high antibody titres and curtailment of infection in animals challenged with strain 78α, it was not statistically significant ($P=0{\cdot}07$ for IgM and 0·4 for IgG).

DISCUSSION

In this, as in our previous study (Johnson *et al.*, 1980), chlamydial infection of the marmoset genital tract induced an acute inflammatory reaction. We now find that inflammation occurs not only after a first infection but following re-infection, although in the one instance that an animal was completely resistant to re-infection, an inflammatory response did not occur. Furthermore, in a number of animals, inflammation persisted after the infection had been eliminated as judged by the failure to isolate chlamydiae. A possible explanation for this persistent inflammation is that chlamydiae were not eliminated but only reduced in number, so that they were difficult to detect. There are, however, two reasons why this is unlikely. First, in every experiment, animals that had apparently cleared their infection had a minimum of 4–6 successive swabs, or more usually 10 or 11, which were consistently negative for chlamydiae. If the persistence of small numbers of chlamydiae in an animal's genital tract was a common occurrence, it would have been expected that swabs would have been positive occasionally, given the large number that were tested. This was generally not our experience as only one animal (D) apparently exhibited this phenomenon. Second, treatment of 4 animals with cyclophosphamide, an immunosuppressive agent that has been shown to exacerbate chlamydial infections in guinea-pigs (Modabber, Bear and Cerny, 1976), had no effect as subsequent swabs were still negative for chlamydiae. Thus, there was no convincing evidence that persistent inflammation was generally due to continued carriage of small numbers of chlamydiae in the genital tract of infected marmosets.

A possible alternative explanation for

persistent inflammation is that antigenic fragments released from dead chlamydiae combine with antibody to form immune complexes which activate complement. There is no evidence at present to support this hypothesis but it merits investigation, particularly in view of the finding that urethral inflammation often persists in patients with chlamydial non-gonococcal urethritis from whom the organisms are not recoverable after antibiotic treatment (Munday *et al.*, 1981). Furthermore, the observations on the marmosets provide a possible explanation for the failure to isolate chlamydiae from some patients with untreated non-gonococcal urethritis. These patients may have chlamydial re-infections in which the organisms stimulate an inflammatory response but then disappear rapidly so that isolation attempts are unsuccessful.

A problem encountered in the present investigation was the relatively small number of animals available for study. The results presented here do not, therefore, lend themselves to extensive statistical analysis. Nevertheless, there is a good indication that despite the failure to prevent recurrent inflammation, immunity to re-infection did develop, as the duration of a second or subsequent infection was generally considerably shorter than that of a primary infection. The apparent failure of animals to resist re-infection with strain 78α in the first re-challenge experiment (Expt. No. 2) may not necessarily indicate lack of immunity, but could be a reflection of inoculating the animals with a possibly overwhelming number of organisms. The minimum number of organisms of each of the two *C. trachomatis* strains required to infect the marmosets was not known because insufficient animals were available to determine this. However, there would seem to have been a considerable excess of organisms of strain 78α, because animals inoculated with strain UW4 received over 10 times fewer organisms and yet they were still infected. Moreover, other workers have been able to infect baboons (Digiacomo *et al.*, 1975), chimpanzees (Jacobs *et al.*, 1978) and grivet monkeys (Møller and Mårdh, 1980; Ripa *et al.*, 1979) with fewer chlamydial organisms than we used.

The mechanism(s) by which the duration of successive infections was curtailed is not known. There was a significant correlation between pre-existing serum IgG antibody at a titre of 1:32 or greater or IgM antibody at a titre of 1:4 or greater and resistance to infection by strain UW4, and a less obvious correlation with strain 78α. However, it would be premature to suggest that serum antibody is an important mediator of protection, since other factors such as local antibody and cell-mediated immunity were not investigated.

It is of interest that the development of immunity to re-infection with *C. trachomatis* was not serotype specific. Thus, infection of animals with strain 78α (serotype D/E) resulted in immunity to re-infection not only with the homologous strain but also with strain UW4, which belongs to an unrelated serotype (serotype H). Furthermore, the serum antibody produced by animals infected with either strain did not exhibit serotype specificity, but reacted at about the same titre with all the serotypes of *C. trachomatis* used as antigens in the antibody test. Thus, the immune response elicited in infected animals was directed not only against strain-specific antigens but also against cross-reacting antigens such as the group-specific and/or the species-specific antigens found in all strains of *C. trachomatis* (Schachter, 1980). It is possible, therefore, that group-reactive antigens stimulate an immune response which is protective. The importance of this concept with regard to human infection is that it may be feasible to vaccinate with several or possibly all serotypes of *C. trachomatis* by using the appropriate cross-reacting antigen(s).

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