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Identification of a New Group of *Chlamydia psittaci* Strains Called TWAR

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A new group of *Chlamydia psittaci* strains has been identified. They are called TWAR after the laboratory designation of the first two isolates. Twelve strains were isolated from pharyngeal swabs of different persons with acute respiratory disease in Seattle, Wash., during 1983 to 1986. One strain was obtained from the eye of a child during the trachoma vaccine study in Taiwan in 1965. Nine strains were characterized in this study. TWAR organisms formed intracytoplasmic inclusions in HeLa cells which were morphologically typical of *C. psittaci* and iodine stain negative (contained no glycogen). Immunological analysis with various chlamydia-specific monoclonal antibodies revealed that TWAR strains belong to the genus *Chlamydia*, are distinct from *C. trachomatis*, and are serologically unique among *C. psittaci*. All TWAR strains so far isolated appear identical serologically. TWAR organisms grew poorly in egg and cell cultures and demonstrated low virulence to mice by intracerebral, intranasal, and intravenous inoculation. Available data suggest that the TWAR strain is a primary human pathogen.

A new group of *Chlamydia psittaci* has recently been identified (14). These organisms were called TWAR after the laboratory designation of the first two isolates, TW-183 and AR-39. Seroepidemiological studies have implicated TWAR agents in several pneumonia epidemics in northern Europe (21, 26, 27). TWAR agents were established as important respiratory pathogens when we succeeded in obtaining a series of isolates from persons with acute respiratory disease (14). We report here the microbiological and immunological characterization of TWAR organisms, their virulence in mice and in monkey eyes, and growth characteristics in eggs and cell culture.

MATERIALS AND METHODS

***Chlamydia* strains.** Nine TWAR strains were studied. They were designated as TW-183, AR-39, AR-59, AR-231, AR-277, AR-388, AR-427, AR-458, and LR-65. Reference *Chlamydia* strains used for comparative studies were *C. trachomatis* A/G-17/OT, B/TW-5/OT, Ba/Ap-2/OT, C/TW-3/OT, D/UW-3/Cx, E/UW-5/Cx, F/UW-6/Cx, G/UW-57/Cx, H/UW-4/Cx, I/UW-12/Ur, J/UW-36/Cx, K/UW-31/Cx, Ll/440/Bu, L2/434/Bu, L3/404/Bu (28, 32, 34), and mouse pneumonitis (24) and *C. psittaci* meningopneumonitis, feline pneumonitis, guinea pig inclusion conjunctivitis, 6BC, PS-1, 33L, Cameron, and W32. Meningopneumonitis (8), feline pneumonitis (1), and guinea pig inclusion conjunctivitis (23) are mammalian *C. psittaci* strains; 6BC (12) and PS-1 are avian psittacosis strains, and both are of parakeet origin. PS-1 was isolated in 1969 in Seattle. Strains 33L (28), Cameron (B. R. Jones, London), and W23 (Australia) are purported to be from humans. All of the above strains were tested for immunological classification by inclusion stains. Selected strains were used in the microimmunofluorescence (micro-IF) test (32) for quantitation of immunological reactivity.

Egg culture. Seven-day-old embryonated chicken eggs were used for yolk sac inoculation (16). Inoculated eggs were incubated at 35°C. Yolk sac membranes were harvested either when the eggs were killed by the growth of chlamydia or, if the eggs survived, on day 12 or 13 before hatching. Smears were then made from the yolk sac membrane on microscope slides, and the chlamydial organisms were identified with modified Macchiavello stain (10) or fluorescein isothiocyanate (FITC) conjugate of TWAR-specific monoclonal antibody when it became available (see below).

Cell culture. Methods for isolation and serial passages of chlamydia in vial culture of HeLa 229 cells and adaptation of chlamydia for bottle cultures have been described (19). Cultures were incubated at 35°C. Cells were pretreated with DEAE-dextran (30 μg/ml) before inoculation (20). Inocula were centrifuged (900 × *g* for 60 min at 22°C) onto cell monolayers (13). Cycloheximide (0.5 μg/ml) was incorporated in the medium for culturing chlamydia (25). Centrifugation was omitted for bottle cultures. Two other cell lines commonly used for chlamydia culture were also studied for their susceptibility to TWAR. They were McCoy and L cells, both mouse cell lines. The vial culture method was used for experiments characterizing inclusions, determining growth enhancement, and comparing cell line susceptibilities.

Inclusion stains. After incubation, inoculated monolayers on 12-mm (diameter) cover slips were fixed with methanol and stained with iodine at 48 h (12, 13), May-Greenwald-Giemsa at 72 h (9), or FITC-conjugated monoclonal antibodies specific to TWAR, the genus *Chlamydia*, or *C. trachomatis* at 72 h.

Monoclonal antibodies. Monoclonal antibodies specific to TWAR were produced by the hybridoma technique of Kohler and Milstein (17) as adapted for chlamydia by our previously published methods (30, 34). Several TWAR-specific monoclonal antibodies were obtained by fusions with TW-183 and AR-39. TWAR specificity was determined by testing monoclonal antibodies in the micro-IF test (32) against 4 TWAR (TW-183, AR-39, AR-277, AR-388), 6 *C. psittaci* (PS-1, meningopneumonitis, guinea pig inclusion conjunctivitis, 33L, Cameron, W23), and 15 *C. trachomatis* serovar strains. Six monoclonal antibodies used in this study were RR-402 from fusion with AR-39, TT-205 from fusion with TW-183 (both TWAR specific), CF-2 and FC-5 (both

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TABLE 1. Characteristics of TWAR inclusions in HeLa cells: comparison with other *Chlamydia* strains

Strains[a]	Response to staining with:				
	Giemsa	Iodine	FA[b]		
			Chlamydia	TWAR	*C. trachomatis*
TWAR	Dense	−	+	+	−
C. psittaci	Dense	−	+	−	−
C. trachomatis	Vacuolar	+	+	−	+

[a] See list in Materials and Methods.
[b] FA, FITC conjugates of monoclonal antibodies specific to the genus *Chlamydia*, TWAR, and *C. trachomatis*.

specific to the genus *Chlamydia*), and KG-5 and LM-8 (both specific to *C. trachomatis*). The latter four monoclonal antibodies were from fusions with *C. trachomatis*. The specificities were determined in the micro-IF test and by enzyme-linked immunosorbent assay (34).

For direct immunofluorescence staining, immunoglobulins were purified from ascitic fluids by using a protein A-Sepharose column (6) and conjugated to FITC by the method of Goding (11), as described in a previous publication (29). Evans blue (0.2%) was used as a counterstain. FITC conjugates were prepared with monoclonal antibodies RR-402, CF-2, and LM-8 for direct immunofluorescence stainings of inclusions. Other monoclonal antibodies were used only for the micro-IF test or enzyme-linked immunosorbent assay.

Mouse virulence test. Four-week-old Swiss Webster mice were inoculated intracerebrally, intranasally, and intravenously with cell-culture-grown TWAR organisms and observed for sickness and death. Each mouse received 3×10^6 (intracerebrally), 4×10^6 (intranasally), or 5×10^7 (intravenously) inclusion-forming units. Control mice were given HeLa cell materials. Five mice in each group were kept for observation for 2 weeks, and another five mice from the same group were sacrificed on day 3 after inoculation to obtain brains, lungs, spleens, and livers. Each organ was divided in half and used separately for histopathological study and infectivity assay in HeLa cell culture (18).

Monkey eye inoculation. Cotton swabs soaked with infected yolk sac suspension were rubbed on the conjunctiva of Taiwan monkeys, and the animals were observed for clinical disease as previously described (31).

RESULTS

TWAR isolates. Thirteen TWAR strains were isolated. Nine of them were serially passed and grown in bottle cultures of HeLa 229 cells. The first TWAR strain, TW-183, was isolated from the eye of a child during a trachoma vaccine study in Taiwan in 1965. The remaining strains (with AR and LR designations) were isolated from the throats of adults with acute respiratory diseases, including pneumonia, bronchitis, and pharyngitis, during the period from April 1983 to March 1986 (14, 15). All patients from whom the organism was isolated developed serological evidence of recent TWAR infections. TW-183 and AR-59 were cultured only in eggs. Cultures of the remaining 11 AR and LR strains were done in both HeLa cells and embryonated eggs. Seven strains were isolated by both culture systems, three by cell culture alone, and one by egg culture alone. Serial passages of TWAR isolates in either egg or cell cultures were not always successful. Three cell culture isolates were adapted directly to grow in bottle culture after 15 to 17 passages in vial cultures. Cell culture adaptation succeeded with the other six strains by inoculation of egg passage 1 or 2 into HeLa cells. In these adaptations, fewer HeLa cell vial culture passages usually were needed. Egg yolk sacs were routinely stained with basic fuchsin (Macchiavello stain), but only 3 of 10 TWAR egg isolates were detected by Macchiavello stain. The remaining seven strains were detected by blind passage to HeLa cells or by direct fluorescent antibody stain of yolk sac smears using TWAR-specific monoclonal antibody.

Inclusion morphology. Intracytoplasmic TWAR inclusions in HeLa cells most closely resembled *C. psittaci* inclusions, which are oval and dense (Giemsa stain) and contain no glycogen (iodine stain). However, TWAR inclusions did not show the variable shape demonstrated by some *C. psittaci* strains. They were clearly different from *C. trachomatis* inclusions, which are vacuolar and contain glycogen.

Immunological classification. The direct fluorescent antibody technique with monoclonal antibodies specific to TWAR, the genus *Chlamydia*, and *C. trachomatis* showed that (i) TWAR inclusions were stained by TWAR- and *Chlamydia*-specific antibodies, (ii) inclusions of other *C. psittaci* strains were stained by *Chlamydia*-specific antibody, and (iii) *C. trachomatis* inclusions were stained by *Chlamydia*- and *C. trachomatis*-specific antibodies (Table 1). The indirect micro-IF test of elementary bodies with TWAR-specific monoclonal antibodies (RR-402 and TT-205) also produced the same reaction patterns, i.e., a positive reaction with TWAR and a negative one with other *C. psittaci* and *C. trachomatis* strains. In addition, all TWAR strains yielded the same titers (1:6,400).

Mouse virulence. TW-183 and AR-39 strains infected mice by three routes of inoculation (intracerebral, intranasal, and intravenous). Infection was proven by recovery on day 3 of organisms from brains with intracerebral inoculation, from lungs with intranasal inoculation, and from spleens, livers, and lungs with intravenous inoculation. The inoculated mice showed weakness within 24 h, probably because of chlamydia toxicity. No apparent symptoms were observed after 24 h. No deaths were observed. Histopathological studies on day 3 after inoculation showed inflammatory reactions in meninges and brain substance, mild interstitial pneumonitis, infiltration in portal areas with foci in liver parenchyma, and hyperplasia of germinal centers and infiltration in both white and red pulp of the spleen. The infiltrating cells were mostly polymorphonuclear leukocytes. Histopathology at other times was not studied. Control mice were negative for isolation and histopathology.

Monkey eye inoculation. Since TW-183 was isolated from eyes, it was inoculated into monkey conjunctivae to see whether conjunctivitis could be produced. Only one of three monkeys inoculated with 10% infected yolk sac suspension into one eye developed mild conjunctival congestion and edema with a duration of 2 to 3 weeks. Follicular conjunctivitis typically seen in Taiwan monkeys inoculated with strains of the trachoma biovar of *C. trachomatis* was not produced (31). Rechallenge of these three monkeys 2 months later with 40% infected yolk sac suspension again caused only mild inflammation in two monkeys with no follicular conjunctivitis. Other TWAR strains were not tested in monkey eyes.

Growth in eggs. TWAR strains demonstrated low virulence in embryonated eggs. None of the TWAR strains, except TW-183, killed eggs during two to three early egg passages. Titrations of HeLa cell-adapted TW-183 and AR-39 strains with HeLa cell titers of 10^8 to 10^9 inclusion-

forming units yielded $10^{4.1}$ and $10^{3.8}$ 50% egg infective doses, respectively.

Growth in cell culture. Enhancement of cell culture growth was studied with egg-grown organisms of TW-183 and AR-39. Individually, DEAE showed variable enhancement, whereas cycloheximide and centrifugation showed more consistent enhancement in the three cell lines tested (Table 2). By far the best enhancement was obtained by centrifugation. When centrifugation was combined with either of the other two treatments, some additive effect was observed with titers 1.5 to 2.5 times greater than with centrifugation alone (data not shown). The best results were obtained by combination of all three treatments (Table 3). Passage experiments showed that incubation of HeLa cell cultures for 3 days yielded higher titers than incubation for 2 to 4 days (3.5 and 12 times, respectively).

DISCUSSION

Immunological data obtained with monoclonal antibodies showed that TWAR strains are chlamydia and that they do not belong in the *C. trachomatis* species. Their inclusion morphology in HeLa cells and their failure to stain with iodine justify classification in the *C. psittaci* species (12, 22). Because of low virulence to eggs, it was not possible to test sulfa drug susceptibility.

Immunological assays indicated that TWAR strains are serologically unique among the *C. psittaci* strains tested. No serological cross-reaction was demonstrated between TWAR and eight other mammalian and avian *C. psittaci* strains (14). So far, all TWAR isolates appear to belong to a single serovar. They all reacted equally with strain-specific monoclonal antibodies made from two isolates (TW-183 and AR-39) separated by time, geographic area, and anatomic site.

Biological characterization revealed that TWAR strains were less pathogenic to mice and chicken embryos and grew less efficiently in cell culture than do avian *C. psittaci* and most mammalian *C. psittaci* and *C. trachomatis* strains (19, 22).

We have established TWAR as an important respiratory pathogen (14). Our study of 386 university students with acute respiratory disease showed that TWAR infection occurred in 12% of students diagnosed with pneumonia (9/76), 5% of those with bronchitis (3/65), and 1% of those with pharyngitis (1/150). Seroepidemiological studies have found TWAR antibody in 25 to 55% of adults in seven widely different areas of the world (33). Antibody was always more common in men than women. It was rare in very young children but increased sharply in prevalence during the ages of 10 to 30 years, and then the high prevalence persisted into old age. The widespread high-incidence epidemics of ornithosis in the Scandinavian countries from 1977 to 1983 has been shown to be largely due to TWAR infection (21, 27). These studies suggest that TWAR is a common cause of infection and may be both endemic and epidemic.

TABLE 2. Enhancement of cell culture growth of TWAR in three different cell lines

Strain[a]	Enhancement	Inclusion count (10^2 IFU/ml)[b]		
		HeLa 229	McCoy	L
TW-183	None	1 (0+)[c]	0+[d]	0
	DEAE	3	0+	0+
	Cycloheximide	3	3	3
	Centrifugation	2,000 (300)	200	5
	All three	4,000[e] (900)	7,000	600
AR-39	None	1 (0+)	0	0
	DEAE	1	0+	0
	Cycloheximide	5	1	1
	Centrifugation	6,000 (200)	80	7
	All three	6,000[e] (600)	500	600

[a] Inocula: 1% infected yolk sac suspensions.
[b] Average of triplicate cover slips from two experiments. IFU, Inclusion-forming units.
[c] Results in parentheses were from inoculation with a 0.1% infected yolk sac suspension.
[d] +, Inclusions found.
[e] Numerous ruptured inclusions not counted.

TABLE 3. Comparative growth of two TWAR strains in three different cell lines

Cell line[a]	Inclusion count (10 IFU/ml)[b]	
	TW-183	AR-39
McCoy	70	270
L	148	259
HeLa 229	6,397	8,467

[a] Growth enhanced by combination of DEAE, cycloheximide, and centrifugation.
[b] Average of triplicate cover slips from two experiments. IFU, Inclusion-forming units. Inocula were 0.1% infected yolk sac suspensions.

Isolation of a strain (IOL-207) similar to TWAR has been reported. Several years after the isolation of TW-183, IOL-207 was isolated in embryonated egg yolk sac from the conjunctiva of an Iranian child with trachoma by workers in England (5). The strain has been described as an atypical, glycogen-negative-inclusion-producing chlamydia which is related to but serologically distinct from *C. psittaci* and *C. trachomatis* and is antigenically similar to TW-183 (5). Antibody to IOL-207 was frequently found in blood donors in London (3) and in school children after 5 years of age in England (2). IOL-207 was shown to induce urethritis and conjunctivitis in baboons (4). Acute conjunctivitis in a laboratory worker was attributed to IOL-207 (7).

A number of findings suggest that the TWAR strain is of human origin and is spread from human to human without a bird or animal host. The usual bird-to-human transmission for *C. psittaci* could not be demonstrated in proven TWAR cases (14) or in the pneumonia epidemics in Finland associated with TWAR antibody (27). The high frequency of TWAR antibody in adults from many areas of the world appears to rule out any bird or animal host except one that is common with worldwide close human contact. Since dogs and cats might meet that requirement, we tested sera from 35 domestic cats and 32 dogs in Seattle, and all failed to show antibody against TWAR antigens in the micro-IF test (33).

For further classification to determine the position of TWAR strains within *C. psittaci* or to justify a new species, we are currently undertaking DNA analysis of TWAR, as well as other *C. psittaci* and *C. trachomatis* organisms.

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