

Lymphogranuloma Venereum

II. Characterization of Some Recently Isolated Strains

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Five *Bedsonia* (*Chlamydia*) isolates from lymphogranuloma venereum (LGV) patients were tested for inclusion type, sulfonamide sensitivity, and mouse virulence. Two matched the classical description of LGV agents. Two were not virulent for mice by the intracerebral route, therefore fitting the description for trachoma-inclusion conjunctivitis agents. One was highly virulent for mice and sulfonamide-resistant, and produced inclusions that did not stain with iodine, all characteristics generally associated with avian bedsoniae. A sixth isolate could not be adequately tested due to poor infective yields. Because of this variety of properties within the *Bedsonia* group, the term LGV might more appropriately be reserved for clinical disease rather than to describe a particular bedsonial agent.

A member of the *Bedsonia* (*Chlamydia*) group of microorganisms is the causative agent of the venereal disease lymphogranuloma venereum (LGV; 2). The seventh edition of *Bergey's Manual of Determinative Bacteriology* refers to the agent as *Miyagawanella lymphogranulomatosis* and describes it as sensitive to sulfonamides, not productive of an iodine-staining inclusion, and virulent for mice when inoculated by the intracerebral (IC) and intranasal (IN) routes but not by the intraperitoneal (IP) route. Many strains of LGV have been isolated (8, 9), but few studies thereof have been reported since the mid-1940's. The only LGV strains extant have a laboratory history of approximately 30 years. In 1967 it was reported that an agent isolated from a patient with clinical LGV had the characteristics of an avian bedsonial strain (sulfa-resistant, highly virulent for mice, and nonproductive of glycogen; 6). This paper presents results of laboratory studies on some bedsonial strains isolated from clinical cases of LGV. Most patients were servicemen and seamen returning from Southeast Asia. Clinical features and results of diagnostic tests for these patients will be published elsewhere (J. Schachter et al., *J. Infect. Diseases*, *in press*).

MATERIALS AND METHODS

Isolation. Agents were isolated from bubo pus by techniques described in an earlier paper (7). Yolk sacs (YS) of embryonated hens' eggs were used, but in some instances mice were inoculated IC. Bubo pus and YS suspensions were tested for bacteriological sterility by inoculating blood-agar plates and thio-

glycollate medium. Specimens were processed in a unit used solely for isolation attempts. No bedsonial agents with the characteristics of these isolates were used in the laboratory.

Serology. Group complement-fixation titers were determined by the methods described by Meyer and Eddie (3), and the presence of group antigen was demonstrated in the isolates by preparing boiled YS antigens.

Titration. (i) Early passage isolates were prepared as a 50% YS suspension in nutrient broth; 1-ml quantities were quick-frozen and stored at -70°C . All titrations were performed on samples from the frozen pool. The egg LD_{50} of the pools was determined by preparing serial 10-fold dilutions of the agents in nutrient broth. Into each of ten 7-day-old eggs, 0.2 ml of a suspension was inoculated. The eggs were incubated at 35°C and candled daily. Embryos which died early (<3 days) were discarded. Deaths were recorded through the 13th day, and the LD_{50} was calculated by the Reed-Muench method (5).

(ii) Serial 10-fold dilutions of the YS-grown agent were used to inoculate mice by the following routes: IC, 0.025 ml; IP, 0.5 ml; and IN, 0.025 ml. The highest concentration tested was a 20% YS suspension. Mice were anesthetized with ether for IC and IN titrations. Dead animals were autopsied, and impression smears were prepared from organs affected (lung, meninges, spleen). Observation periods were 14 (IC) and 21 days (IP or IN).

Tissue culture. Monolayers of L929 and HeLa 229 cells were prepared on cover slips in Leighton tubes by planting 150,000 cells and incubating for 3 days in the presence of a complete medium containing Eagle Minimum Essential Medium plus 10% inactivated calf serum. The monolayers were infected with 1% YS suspensions of bedsonial agent for 2 hr at room temperature. The sheets were then washed with

Hanks balanced salt solution and incubated at 35 C in the presence of a maintenance medium of Eagle Minimum Essential Medium and 2% serum, supplemented with 0.1% glucose. Cover slips were removed at intervals for staining.

Staining techniques. For the iodine stain, cell monolayers on cover slips were fixed in methanol for 5 min, dried, and stained with Lugol solution for 5 min. The Lugol solution was then washed off with tap water and the preparations were examined as wet mounts. The same cover slips were then decolorized in methanol, dried, and stained with Giemsa (3).

A modified Macchiavello technique (3) was used to stain both YS and mouse-organ smears. Dead animals were included in titrations only when smears of their organs contained elementary bodies.

Sulfadiazine sensitivity. Isolates were titrated in ovo. The effect of sulfadiazine was measured by inoculating 0.1 ml of sterile distilled water containing 1 mg of sulfadiazine into the YS of eggs immediately after the agent was inoculated. The LD₅₀ values with and without sulfadiazine were determined in parallel.

Isolates tested. The isolates used, the passage level tested, and the year of isolation, respectively, were as follows: 33-L, YS-7, 1964; 404-B, YS-4, 1967; 434-B, YS-5, 1967; 440-L, YS-4, 1967; 470-L, YS-4, 1968. Another isolate, 420-L (1967), was also tested, but because of its poor growth in the YS a stable pool could not be prepared.

RESULTS

All agents used were previously isolated in the YS of embryonated hens' eggs. Isolation attempts in mice failed. Isolates were identified as *Bedsonia* by serial passage of characteristic elementary bodies and by demonstrating group antigen in YS. The complement-fixing antigen titers were 1:256 or higher. All isolates except 420-L were regularly lethal for eggs, and large YS pools could be prepared to perform comparative tests on the same material. Lethality was irregular for isolate 420-L, and only occasionally did all eggs in an experiment die at a 50% inoculum, so that a pool could not be prepared. Isolate 33-L was highly virulent for eggs, with an LD₅₀ of 10⁸ or greater. The infectious and lethal doses of this isolate were essentially the same; surviving embryos seldom gave positive test results for the agent. Other isolates tested (404, 434, 440, and 470) were moderately virulent for the egg embryo, with LD₅₀ values of about 10⁴ to 10⁵, and their infectious doses exceeded their lethal doses by approximately 2 log. YS smears and suspensions from surviving embryos were often as heavily infected as those prepared from dead embryos.

Table 1 shows the results of titrations in mice. Isolate 33-L was lethal and had a high titer by all routes tested. Isolates 434 and 440 were lethal, with low titers, when inoculated by the IC and IN routes but were not lethal by the IP route.

TABLE 1. Titrations of LGV isolates in eggs and mice

Isolate	YS	Mice		
		IC	IN	IP
33-L	8.0 ^a	8.5	6.5	8.0
404-B	3.8	X ^b	X	X
420-L	<1.0	X	X	X
434-B	4.2	2.5	2.2	X
440-L	4.5	2.3	1.5	X
470-L	5.5	X	X	X

^a Log₁₀ egg LD₅₀/g of YS.

^b X = No deaths at 20% YS suspension.

The remaining isolates were not lethal by any route tested, although isolates 404 and 470 produced gross pathology in the surviving animals, whereas 420-L did not. The survivors became carriers, as the agents could be recovered from brains and lungs. Serial mouse passage (with early YS-passage pools) of these two agents did not establish strains lethal for mice. Later YS passage of isolates 404 and 470 occasionally resulted in a higher egg LD₅₀ (10⁵ to 10⁶) than in earlier passages. This later-passage material, when screened by mouse inoculation, sometimes was lethal after IC inoculation. In these instances, deaths usually occurred in 48 hr and could have been toxic.

All isolates except 420-L grew well in tissue culture and were serially transferable in cells. The infectivity by inclusion count was usually three to five times higher in HeLa cells than in L cells. Isolate 33-L had a rapid cytopathic effect and produced inclusions that did not take the iodine stain. Isolates 404, 434, 440, and 470 all produced iodine-staining inclusions (giving positive results between 24 and 36 hr) that were observed within vacuoles. Of these isolates, 404 produced the least cytopathic effect, with a heavily infected (60%) monolayer often surviving for 7 or more days. With the other isolates, a similar infection level destroyed the monolayers in 72 hr.

Table 2 shows the results of sulfadiazine sensitivity tests. Addition of 1 mg of sulfadiazine to each egg did not affect isolate 33-L but completely suppressed the lethal effect of other agents tested. Isolate 420-L was not tested for sulfadiazine sensitivity for lack of a suitable end point.

DISCUSSION

The typical description of the LGV agent, regarding mouse virulence, sulfonamide sensitivity, and inclusion type, is based upon work performed 20 to 30 years ago or on studies of an

TABLE 2. Effect of sulfadiazine on LGV isolates

Isolate	Egg LD ₅₀	
	No sulfadiazine	Sulfadiazine added (1 mg/embryo)
33-L	8.0 ^a	8.0
404-B	4.5	X ^b
434-B	4.2	X
440-L	5.2	X
470-L	5.5	X

^a Log₁₀ egg LD₅₀/g of YS.

^b X = No deaths at 20% YS suspension.

LGV isolate (strain JH) having a 30-year history of laboratory passage. The present study of six recent low-passage isolates reveals some differences in their properties. All six isolates were derived from patients with classical LGV; five were from bubo aspirates and the sixth was from an excised perirectal lymph node. Isolate 420-L, an extremely feeble grower in YS, did not grow at all in the other systems tested. The characteristics of isolate 33-L were those usually associated with some avian bedsoniae, that is, highly virulent for mice (3), iodine-negative, and sulfonamide-resistant (1, 4). Such agents are not known to be prevalent in the clinical syndrome, but they could provide one explanation for the sporadic failures of sulfonamide treatment. It need not be the only explanation, for tetracycline treatment sometimes also fails, and all these isolates are sensitive to tetracycline.

The characteristics of isolates 434 and 440 are identical to those of the prototype JH strain of LGV [which produces an iodine-staining inclusion (1), in contradiction to the description given in *Bergey's Manual*]. Isolates 404 and 470, however, differ from the prototype in mouse virulence. The danger of using pathogenicity patterns for isolate typing is well known, but in the absence of more refined markers, mouse virulence is often used for this purpose. This marker is particularly important in the differential identification of trachoma-inclusion-conjunctivitis (TRIC) agents, bedsoniae which may be sexually transmitted in man. A bedsonia isolated from the human genital tract is generally considered as an LGV agent if it kills mice inoculated by the IC route and as a TRIC agent if it does not. Therefore isolates 404 and 470 would have been considered as TRIC agents had they been derived from the urethra and not from an inguinal bubo. The only other distinctions are the ability of TRIC agents to produce follicular conjunctivitis in primates (LGV agents presumably do not) and the cytopathic effects and rapid growth seen

in tissue culture with these LGV agents. A test in primates would be impractical for routine use with all bedsonial isolates derived from the human genital tract. The pathogenicity for the conjunctiva of these LGV isolates is being studied.

The original attempts at isolating LGV agents in YS resulted in at least one isolate obtained in ovo that could not be maintained in mice (8). The mouse was the most popular indicator host in early studies of LGV. Obviously, agents not lethal for mice could not have been isolated by this method. Studies like that of Wall (9), showing the mouse superior to the egg for isolation, may have reflected the properties of the agents prevalent in a particular area at a specific time. Apparently LGV agents need not be virulent for mice; therefore, a major criterion for differentiating between LGV and TRIC agents is lost. Possibly there are no valid differences, for there may be overlap within the groups if enough isolates are tested.

LGV is a clinical entity, and the term should not be used to refer to a single type of *Bedsonia*. Possibly any *Bedsonia* may produce this clinical entity, but certainly the agents isolated from LGV patients in this study present a spectrum of qualities within the *Bedsonia* group.

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