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DIFFERENTIATION OF PATHOGENIC AND SAPROPHYTIC LEPTOSPIRES WITH 8-AZAGUANINE

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ABSTRACT

JOHNSON, RUSSELL C. (University of Minnesota, Minneapolis), AND PALMER ROGERS. Differentiation of pathogenic and saprophytic leptospires with 8-azaguanine. J. Bacteriol. **88**:1618–1623. 1964.—The use of the purine analogue, 8-azaguanine, as a differential agent for the separation of pathogenic and saprophytic leptospires was investigated. Growth of strains of the saprophyte *Leptospira biflexa* was almost insensitive to the bacteriostatic action of 8-azaguanine at concentrations varying from 25 to 600 μg/ml; these saprophytic leptospires were serially transferred five times in media containing 225 μg without any change in growth rate or cell yield. In contrast, decreased growth rate and cell yield of the pathogenic serotypes were observed with 25 to 50 μg/ml of 8-azaguanine. Complete inhibition of growth occurred at concentrations of 100 μg/ml and above. A medium containing 225 μg/ml of 8-azaguanine was successfully used to differentiate 20 serotypes of pathogenic leptospires and 10 saprophytic strains. *L. andaman* CH11, *L. semarang* Veldrat S1 73, and *L. andaman* Correa, were classified with the *L. biflexa* strains on the basis of their growth response to 8-azaguanine.

Saprophytic leptospires differ from the pathogenic strains culturally, serologically, and in their inability to infect mammals. Hindle (1925) found that the leptospires he isolated from London tap water would grow in a feces medium, whereas pathogenic strains would not. This observation was confirmed by Walker (1927). Recently, several methods of differentiating saprophytic and pathogenic leptospires have appeared in the literature. Füzi and Csóka (1960) found that the growth of pathogenic leptospires is inhibited by the presence of 10 ppm of copper sulfate in the culture medium, whereas the growth of the saprophytes was not. Also, they reported that the saprophytic strains have greater oxidase activity (Füzi and Csóka, 1961*b*), and they decompose egg yolk faster than do the pathogenic strains (Füzi and Csóka, 1961*a*). Yanagawa, Hiramune, and Akaike (1963) found that colonial growth in the presence of CO_2-free air could be used for the partial classification of pathogenic and saprophytic leptospires.

In our investigation of purine and pyrimidine utilization by leptospires, we found that purines were utilized, whereas pyrimidines were not (Johnson and Rogers, 1964*b*). This observation was extended by use of purine and pyrimidine analogues. None of the pyrimidine analogues tested inhibited growth, but some of the purine analogues markedly inhibited growth. The exception to this observation was the saprophyte, *L. biflexa*, which was not inhibited by any of the purine analogues tested.

This report is concerned with the presentation of a simple method for differentiating pathogenic and saprophytic leptospires based upon selective growth inhibition by the purine analogue, 8-azaguanine.

MATERIALS AND METHODS

Twenty strains of pathogenic serotypes of leptospires and 10 strains of saprophytic leptospires were used in this investigation. Seventeen strains of pathogenic serotypes and eight strains of saprophytic leptospires were provided by A. D. Alexander, Walter Reed Army Institute for Research. *Leptospira pomona* Wickard was obtained from J. B. Wilson, Department of Bacteriology, University of Wisconsin, and one strain each of *L. icterohaemorrhagiae* and *L. canicola* was obtained from D. E. Low, Department of Veterinary Medicine, University of Minnesota. *L. biflexa* Wa Reiden and *L. biflexa* Gent were received from Mildred M. Galton, Communicable Disease Center, Atlanta, Ga.

Stock cultures of leptospires were maintained in a basal medium containing 10% pooled rabbit serum, 0.02 M Na_2HPO_4-KH_2PO_4 buffer (pH. 7.4)

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(Johnson and Wilson, 1960), 10^{-3} M NH_4Cl, and 5 μg/ml of thiamine (Johnson and Gary, 1963). Cultures were incubated at 30 C, and transferred every 7 days. 5-Fluorouracil (100 μg/ml) was incorporated in the basal medium to control any contamination that might have occurred (Johnson and Rogers, 1964*a*).

8-Azaguanine was obtained from Calbiochem. The final concentrations of 8-azaguanine in the growth tubes varied from 25 to 1,000 μg/ml. The analogue was sterilized with basal medium minus the serum by autoclaving (20 min at 121 C).

Growth was measured by light-scattering with a Coleman (model 7) photonephelometer as described by Johnson and Gary (1962). The relationship between nephelometer readings and cell counts remained constant, except at the high nephelometer reading obtained with the saprophytic strains. Samples from each experiment, and especially those using the saprophytic strains, were counted with a Petroff-Hausser counting chamber to verify the relationship between the cell count and the nephelometer reading. The inoculum used (0.1 to 1%, v/v) yielded an initial cell count of approximately 2×10^6 organisms per milliliter.

RESULTS

As previously reported (Johnson and Rogers, 1964*a*), leptospires were resistant to the action of the pyrimidine analogue, 5-fluorouracil. Further studies with the pathogen *L. pomona* (Johnson and Rogers, 1964*b*) demonstrated its sensitivity to some of the purine analogues. This observation was extended to include other pathogenic serotypes and one strain of the saprophyte *L. biflexa* (Table 1). 8-Azaguanine and 6-mercaptopurine inhibited the growth of *L. pomona*, *L. canicola*, and *L. australis* A, but had little effect on the growth of the saprophyte. 2-Aminopurine and the pyrimidine analogues had little or no inhibitory action on these spirochetes. Because 8-azaguanine is more soluble than 6-mercaptopurine, further investigations were restricted to 8-azaguanine.

The pathogen *L. pomona* Wickard was grown in the presence of increasing concentrations of 8-azaguanine (Fig. 1). At concentrations of 25 and 50 μg/ml, a decreased rate of growth and cell yield was observed; at 100 to 225 μg/ml, inhibition of growth was virtually complete. Upon closer examination of growth in these latter concentrations, it was determined that one to two generations occurred before the bacteriostatic action of the analogue was manifested. This increase in cell number was not detected with the

TABLE 1. *Growth response of leptospires to pyrimidine and purine analogues*

Test organism	Addition to basal medium*					
	None	5-Fluorouracil	5-Bromouracil	8-Azaguanine	6-Mercaptopurine	2-Aminopurine nitrate
L. pomona Wickard	106†	99	93	2	10	85
L. canicola Hond Utrecht IV	90	73	79	0	0	90
L. australis A Ballico	58	68	63	0	0	41
L. biflexa‡	84	65	74	70	60	75

* Basal medium: 10% rabbit serum, 5 μg/ml of thiamine, 10^{-3} M NH_4Cl, and 0.02 M NaK phosphate (pH 7.4). Initial count was 2×10^6 to 4×10^6 cells per ml; incubation was at 30 C for 7 days. Concentration of analogues was 100 μg/ml.

† Results expressed as increase in nephelometer reading.

‡ Strain unknown.

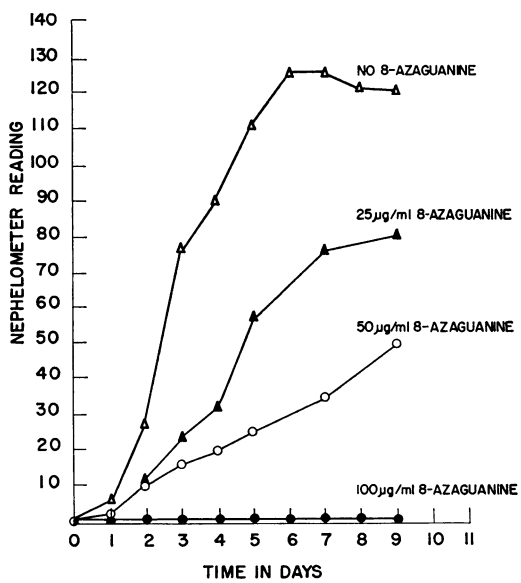

FIG. 1. *Growth response of Leptospira pomona Wickard to increasing concentrations of 8-azaguanine in basal medium.*

nephelometer or the unaided naked eye when a small inoculum (0.1 to 1%, v/v) was used. When a large inoculum (5%, v/v) was used, an increase in the nephelometer reading was observed which reflected one to two generations. After this initial increase in the nephelometer reading occurred, no further increase was observed (Fig. 2A). As shown in Fig. 2B, transfer of the organisms grown in 8-azaguanine into fresh medium resulted in normal growth in the absence of the analogue, and complete inhibition of growth in the presence of 25 μg/ml. Thus, under these conditions, the action of 8-azaguanine on leptospires was bacteriostatic.

In contrast to the results obtained with *L. pomona*, no observable difference in the growth profile of the saprophyte, *L. patoc*, occurred when grown with 25 and 50 μg/ml of azaguanine. At 250 μg/ml, a very slight decrease in cell yield was seen (Fig. 3). The highest concentration of 8-azaguanine tested was 1 mg/ml which only resulted in a 30% reduction in cell yield. In addition, the growth of *L. patoc* was initiated with an inoculum of 100 organisms per ml in media containing 225 μg/ml of the analogue, and transferred serially five times in the medium without any detectable change in growth rate or cell yield. The initial experiments with three pathogenic strains and one saprophytic strain of leptospires suggested that these two groups of organisms could be separated on the basis of their sensitivity to the bacteriostatic action of 8-azaguanine. To ascertain whether this premise was valid, 17 additional pathogenic serotypes and 10 saprophytic strains of leptosipres were tested

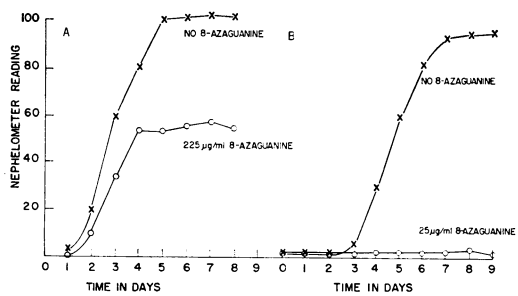

FIG. 2A. *Growth response of large inoculum (5%, v/v) of Leptospira pomona Wickard in basal medium containing 225 μg/ml of 8-azaguanine.*

FIG. 2B. *Transfer of Leptospira pomona Wickard from basal medium containing 8-azaguanine (225 μg/ml) to basal medium with and without 25 μg/ml of 8-azaguanine.*

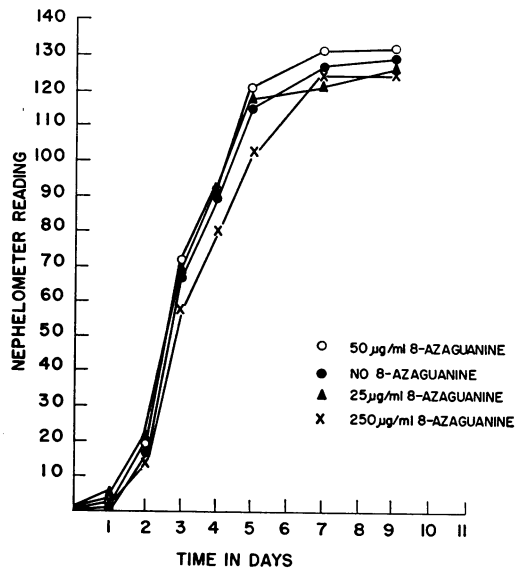

FIG. 3. *Growth response of Leptospira patoc Patoc I (see footnote d, Table 2) to increasing concentrations of 8-azaguanine in basal medium.*

(Table 2) in media with and without 8-azaguanine 225 μg/ml). All the saprophytic leptospires grew well in the presence and absence of 8-azaguanine. The pathogenic leptospires grew well in the basal medium without 8-azaguanine, but their growth was almost completely inhibited when 225 μg/ml of the analogue were present. No additional growth of these serotypes was observed upon prolonged incubation in the medium containing 225 μg/ml of 8-azaguanine. Thus, the total population of pathogenic leptospires appears to be uniformly sensitive to the action of 8-azaguanine. Changes in the serum concentration apparently had no effect upon the inhibitory action of 8-azaguanine. *L. butembo*, *L. celledoni*, and *L. grippotyphosa* were tested for growth in the presence and absence of 225 μg/ml of 8-azaguanine in the basal medium containing 10 and 20% rabbit serum. Growth of these serotypes was equally inhibited by 8-azaguanine in both media.

DISCUSSION

The differentiation of pathogenic and saprophytic leptospires is important for both taxonomic and diagnostic purposes. At the present time, the biological method of choice for differentiation is the oligodynamic action of copper ions in the growth medium. Saprophytic strains

are less sensitive to inhibition by copper than are the pathogenic serotypes.

Shiga (1924), Anjow (1928), and Uhlenhuth (1948) successfully used the copper ion test to separate these two groups of leptospires. However, Sardjito (1931) and Hirsch (1931) were unable to achieve satisfactory separation of these organisms with this method. Füzi and Csóka (1960) refined the copper ion test, and studied the growth response of 61 pathogenic serotypes and 13 saprophytic strains of leptospires using this method. They found that saprophytic strains of leptospires would grow in culture media containing 10 or 1 ppm of copper sulfate. In contrast, the pathogenic strains were inhibited by 10 ppm of copper sulfate, but not by 1 ppm. Unfortunately, this method of differentiation has not been consistently successful when used by other investigators. This difficulty may be largely due to variation in the chelating capacity of some of the media used, thus altering the effective concentration of free copper ions in the medium.

The method of differentiation proposed in this paper is as simple and fast as the copper sulfate test. The use of 225 μg/ml of 8-azaguanine resulted in a clear distinction between all saprophytic and pathogenic leptospires tested so far in this laboratory. In addition, the concentration of 8-azaguanine used is more than twice the concentration required to bring about maximal inhibitory growth of the pathogenic serotypes, but still is one-fourth the concentration required to produce any significant growth inhibition of the saprophytes. Also, variation in the serum concentration in the medium did not alter the results obtained. We have observed (Johnson and Rogers, *unpublished data*) that purines do not reverse the inhibitory effect of 8-azaguanine when it is used at 225 μg/ml. For unequivocal results, an inoculum of 0.1 to 1% is recommended. Leptospires usually grow one to two generations before the action of 8-azaguanine is apparent, as is the case with other microorganisms (Smith and Matthews, 1957). Thus, by using a small inoculum with the 8-azaguanine medium, the difference in growth response between the pathogenic and saprophytic leptospires is maximal.

It is possible that saprophytic leptospires can be separated into at least two groups on the basis of biological differences. Babudieri (1960) separated nonpathogenic leptospires into two groups

TABLE 2. *Effect of 8-azaguanine on the growth of saprophytic and pathogenic leptospires*

Test organism	Addition to basal medium[a]	
	None	225 μg/ml of 8-azaguanine
Pathogenic strains		
L. pomona Wickard	88[b]	3
L. pomona Pomona	86	1
L. icterohemorrhagiae[c]	89	2
L. icterohemorrhagiae M20	67	2
L. canicola[c]	57	1
L. canicola Hond Utrect IV	100	0
L. butembo Butembo	65	4
L. celledoni Celledoni	38	2
L. bataviae Swart	69	3
L. djasiman Djasiman	75	1
L. hyos Mitis Johnson	55	2
L. autumalis Fort Bragg	55	2
L. ballum Mus 127	54	0
L. pyrogenes Salinem	96	2
L. alexi HS616	65	1
L. grippotyphosa Moskva V	48	2
L. boricana HS622	59	1
L. wolffi 3705	78	2
L. javanica Veldrat, Batavia 46	58	2
L. australis A Ballico	80	1
Saprophytic strains		
L. semerang Veldrat S173	125	119
L. patoc Patoc I[d]	121	108
L. sao-paulo Sao Paulo[d]	125	113
L. biflexa Lt430	114	106
L. biflexa CDC	115	90
L. biflexa Waz	136	130
L. andaman A CH11	130	114
L. andaman Corea	127	116
L. biflexa Wa Rieden	120	109
L. biflexa Gent	127	111

[a] Basal medium: 10% rabbit serum, 10^{-3} M NH_4Cl, 5 μg/ml of thiamine, and 0.02 M NaK phosphate buffer (pH 7.4). Incubation 5 to 7 days at 30 C. Initial count was 10^6 to 5×10^6 cells per ml.

[b] Results expressed as increase in nephelometer reading.

[c] Strain unknown.

[d] International Committee on Bacteriological Nomenclature. Minutes of a meeting of the taxonomic subcommittee on *Leptospira*. Annex II. Montreal, 16–17 August 1962.

on the basis of vitamin B_{12} requirements. Yanagawa et al. (1963) found a difference among the saprophytic strains in their CO_2 requirement for colonial growth. A preliminary experiment with 8-azaguanine indicates some variation of response within the pathogenic and saprophytic groups of leptospires to the bacteriostatic action of this analogue. For example, the growth of *L. icterohemorrhagiae* and *L. canicola*, maintained by animal passage, was inhibited by 25 μg/ml of 8-azaguanine. The growth of *L. pomona* and *L. australis* A, maintained in rabbit serum medium, was not inhibited at this concentration of 8-azaguanine. Between 50 and 100 μg/ml of the analogue were required for maximal growth inhibition of these strains.

The classification of several serotypes of leptospires as pathogens has recently been reevaluated. Studies on the biological properties of *L. semaranga* and *L. andaman* A, by Füzi and Csóka (1960, 1961*a*, *b*) and Yanagawa et al. (1963), demonstrated that they belong with the *L. biflexa* strains. Füzi and Csóka (1961*c*) reported that these two serotypes had no virulence for laboratory animals, and were serologically related to *L. biflexa*. The investigations of Babudieri and Dymowska (1961) disclosed that *L. semaranga* was serologically located within the *L. biflexa* group. Our results with the 8-azaguanine test also place these two serotypes with the *L. biflexa* strains. In addition, *L. andaman* Correa, which was reportedly isolated from the spinal fluid of a man, responds to the 8-azaguanine test in the same manner as the saprophytes. It is also related to the *L. biflexa* group serologically (A. D. Alexander, *personal communication*).

The site and mode of action of 8-azaguanine in the pathogenic leptospires, and the reason for the relative insensitivity of the saprophytic strains to this analogue, are currently under investigation. Although the purine analogues are quite toxic to animals, their use alone, and in conjunction with antibiotics for the treatment of leptospirosis, merits some investigation.

ACKNOWLEDGMENTS

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