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Purine Analogue Sensitivity and Lipase Activity of Leptospires

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The genus *Leptospira* can be divided into three groups based on purine analogue sensitivity and lipase (trioleinase) activity. Group 1 contains members of the "parasitic complex" of leptospires which initially cannot grow in media containing 10 μg of 2,6-diaminopurine (DAP) per ml or 200 μg of 8-azaguanine per ml. In addition, leptospires in this group possess lipase activity. Group 2 also contains members of the "parasitic complex" of leptospires. Although these leptospires are similarly sensitive to 8-azaguanine, they differ from group 1 leptospires in that they grow in media containing 10 μg of DAP per ml, and they do not possess detectable lipase activity. Group 3 consists of leptospires belonging to the "biflexa complex." These leptospires are resistant to both purine analogues and have lipase activity.

The recent report of a World Health Organization expert group recommended that the genus *Leptospira* be considered as monospecific until it is possible to circumscribe species with confidence (1). The specific epithet "*interrogans*" was recommended as the type species. However, it was recognized that at least two main complexes of the genus *Leptospira* appeared to exist. The "parasitic complex" represents the nutritionally fastidious strains which are parasitic in some vertebrates and pathogenic for others. The other complex, "biflexa complex," is largely comprised of the strains isolated from water and, because no reservoir hosts are known for them, they are often referred to as "saprophytic" (16).

Evidence is accumulating which indicates that the genus *Leptospira* consists of more than two complexes. Cross-immunity studies with virulent leptospires suggest the "parasitic complex" is comprised of more than one group (9). The report of Haapala et al. (*unpublished data*), which deals with deoxyribonucleic acid (DNA) duplexing studies indicates that the genus contains at least four genetically different groups of leptospires. This report is concerned with the characterization of three biologically different groups of leptospires and a rapid means of identifying these groups.

MATERIALS AND METHODS

We used 74 strains of leptospires in this investigation. A majority of the cultures were provided by A. D. Alexander (Walter Reed Army Institute of Research). Cultures were also obtained from the late Mildred M. Galton (National Communicable Disease Center, Atlanta, Ga.), O. H. V. Stalheim (National Animal Disease Laboratory, Ames, Iowa), and R. Crawford (University of Iowa, Iowa City). The identity of serotypes *borincana* and *alexi* was confirmed by using specific antisera also obtained from A. D. Alexander. The classification system of leptospires used in this report was recommended by a World Health Organization expert group (1).

Cultures were maintained in 10% heat-inactivated rabbit serum medium (5) at 30 C. A Tween 80-albumin medium was used for the purine analogue and lipase studies. This medium is a modification of that described by Ellinghausen and McCullough (3) and was prepared as previously described (5). Cells used for purine analogue studies were from cultures in the log or early stationary phase of growth. Unless stated otherwise, an inoculum which yielded approximately 3×10^7 organisms per ml in the test medium was used in the 2,6-diaminopurine (DAP) sensitivity assays. This is equivalent to a 10% (v/v) inoculum. A 1% (v/v) inoculum was used to determine 8-azaguanine sensitivity. Growth was measured daily with a Coleman model 7 photonephelometer calibrated with an arbitrary turbidity standard (14). The purine analogue sensitivity assays were terminated after an incubation period of 4 to 6 days, or when the control culture reached the late log phase or the early stationary phase of growth. The relationship between nephelometer reading and cell number was verified by periodic counts with a Petroff-Hausser counting chamber. Nephelometer readings of 10 and 100 represent 3.4×10^7 and 48×10^7 cells per ml, respectively.

The DAP, adenine, and guanine used were purchased from Calbiochem (Los Angeles, Calif.), and solutions of these compounds were sterilized by filtration. Triolein was purchased from the Hormel Institute, Austin, Minn. ^{14}C-*8*-adenine was obtained from New England Nuclear Corp., Boston, Mass.

To assay for the incorporation of adenine, cells were grown in medium containing 15 μg of ^{14}C-labeled adenine per ml. At 3, 4, and 5 days postinoculation, cells from 5 ml of the test medium were harvested by centrifugation (20 min at 12,000 × *g*), washed twice in 0.01 M $NaKPO_4$ buffer (*p*H 7.4), and resuspended in distilled water. A portion of the cell suspension was plated at infinite thinness on glass planchets and radioactivity was measured with a gas-flow counter.

Lipase activity was determined in the following manner. An 0.1-ml amount of triolein was dissolved in 30 ml of ethyl alcohol. One volume of this solution was added to 20 volumes of sterile 1% bovine albumin in 0.01 M $NaKPO_4$, *p*H 7.4. The triolein was layered on the surface of the 1% albumin and mixed. The degree of turbidity obtained was directly related to how well the triolein solution was layered on the 1% albumin before mixing. The triolein-albumin mixture was prepared and used on the same day. Stationary phase leptospire culture (1 ml) was added to a tube containing 9 ml of triolein-albumin mixture, and the decrease in turbidity was monitored with the nephelometer. If a 50% reduction of turbidity did not occur within 8 hr, the test was repeated with 5 ml of culture supernatant fluid added to 5 ml of triolein-albumin mixture. A culture was considered lipase-negative when 5 ml of culture supernatant fluid did not cause a reduction in turbidity within 72 hr. The assay was done at room temperature and a pancreatic lipase (pig) preparation was used as a standard for each test. Leptospiral lipase activity is expressed as relative units. A relative unit of leptospiral lipase is equivalent to a unit of pig pancreatic lipase (Schwartz Bio Research Inc., Orangeburg, N.Y.), based on the time required to produce a 50% reduction of turbidity of the triolein-albumin emulsion. One unit of the pig pancreatic lipase produces 1 μmole of acid per min at 25 C with olive oil emulsion as the substrate. The 50% reduction in turbidity of the triolein-albumin emulsion produced by 1.8, 3.6, and 7.2 units of pancreatic lipase occurred in 47, 25, and 12 min, respectively.

TABLE 1. *Group 1 leptospires ("parasitic complex")*

Serogroups, serotypes, strains	Relative units[a] of lipase activity
Icterohaemorrhagiae	
icterohaemorrhagiae W39 (1)[b]	1.7
copenhageni M20	1.5
mankarso Mankarso (3)[b]	13.8
Canicola	
canicola Hond Utrecht IV (2)[b]	2.5
malaya Mal 108	3.7
Pyrogenes	
pyrogenes Salinem	7.4
hamptoni 29	2.5
Cynopteri	
butembo Butembo	0.3
Autumnalis	
rachmati Rachmat (1)[b]	41.4
fort-bragg Fort Bragg	1.3
djasiman Djasiman	4.7
Australis	
australis Ballico	1.5
Pomona	
pomona Pomona (2)[b]	2.4
Grippotyphosa	
grippotyphosa Mal 1540	5.5
Hebdomadis	
wolffii 3705 (1)[b]	11.0
Bataviae	
paidjan Mal 1415	4.8
bataviae Swart	7.5

[a] Relative units of lipase activity per volume of culture medium containing 10^8 leptospires. All figures to be multiplied by 10.

[b] Number of strains tested with similar results.

TABLE 2. *Group 2 leptospires ("parasitic complex")*

Serogroups, serotypes, strains	Increase in cell no. per ml[a]	
	Control	10 μg of DAP/ml
Icterohaemorrhagiae		
sarmin Sarmin	33	30
Javanica		
javanica Veldrat Bataviae 46 (3)[b]	38	32
poi Poi	34	30
sorex-jalna Sorex-Jalna	47	42
coxi Cox	25	25
sofia Sofia 874	38	38
Celledoni		
celledoni Celledoni	33	33
whitcombi Whitcomb	43	38
Ballum		
ballum Mus 127 (3)[b]	31	30
castellonis Castellon 3	49	43
arboreae Arborea	17	15
Pyrogenes		
alexi HS616	29	28
Cynopteri		
cynopteri 3522C	37	35
Autumnalis		
autumnalis Akiyami A	48	45
Hebdomadis		
worsfoldi Worsfold	40	39
jules Jules	45	42
borincana HS622	42	36
saxkoebing Mus 24	34	34
Tarassovi		
tarassovi Perepelicin	40	38
bakeri LT 79	19	17
atlantae LT 81	28	24
bravo Bravo	14	14
chagres LT 924	26	21
kisuba Kisuba	24	22
rami LT 955	20	20
atchafalaya LSU1013	25	23
gatuni LT 839	25	24
guidae RP29	23	20

[a] All figures to be multiplied by 10^7.

[b] Number of strains tested with similar results.

TABLE 3. *Group 3 leptospires ("biflexa complex")*

Serogroups, serotypes, strains	Increase in cell no. per ml[a]			Relative units[b] of lipase activity
	Control	10 μg of DAP/ml	200 μg of 8-azaguanine/ml	
Semaranga				
semaranga Veldrat Semarang 173	72	72	65	8.2
patoc Patoc I	72	69	70	1.7
sao-paulo Sao Paulo	63	60	58	2.7
Andamana				
andamana CH 11	35	31	29	0.9
andamana Correo	62	59	62	0.7
Biflexa				
biflexa A-284	57	52	46	3.5
biflexa CDC	53	50	43	1.0
biflexa I-65-1	59	50	44	1.1
biflexa I-65-5	54	54	51	2.4
biflexa Lt 430	96	89	82	6.2
biflexa Lt 965	89	33	72	0.1
biflexa Lt 1120	62	60	22	3.6
biflexa Waz	54	19	46	7.9
biflexa Gent	41	39	39	5.0

[a] All figures to be multiplied by 10^7.

[b] Relative units lipase activity per volume culture medium containing 10^8 leptospires. All figures to be multiplied by 10.

RESULTS

The "parasitic complex" of leptospires appeared to be a homogeneous group based on sensitivity to the purine analogue, 8-azaguanine. None of the leptospires listed in Table 1 and Table 2 grew in the presence of 200 μg of 8-azaguanine per ml. However, members of this complex varied in their sensitivity to another purine analogue, DAP. Based on this difference in DAP sensitivity, these leptospires were separated into two groups. The leptospires of the "parasitic complex," which initially were unable to grow in 10 μg of DAP per ml, were placed in group 1 (Table 1). Leptospires placed in group 2 were resistant to DAP. These leptospires grew to approximately the same extent in the presence or absence of 10 μg/ml DAP (Table 2). In addition to the difference in DAP sensitivity, another biological characteristic could be correlated with these two groups of "parasitic" leptospires. Leptospires located in group 1 (DAP and 8-azaguanine-sensitive) possessed lipase (trioleinase) activity (Table 1). Group 1 whole cultures or culture supernatant fluids produced a clarification of an albumin-triolein emulsion. Group 2 leptospires (DAP-resistant, 8-azaguanine-sensitive) did not possess detectable lipase activity (Table 2). These cultures failed to cause a reduction in the turbidity of the triolein emulsion after reaction periods of as long as 9 days.

The leptospires of the "biflexa complex" were placed in group 3. They were resistant to both 8-azaguanine and DAP and possessed lipase activity (Table 3).

Serotype *kabura* was the only leptospire en-

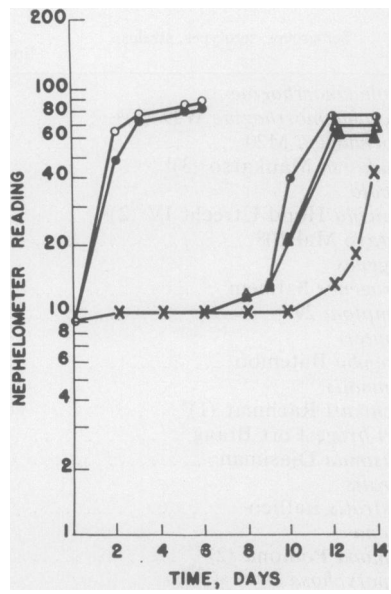

FIG. 1. *Growth response of serotype pomona Wickard to 2,6-diaminopurine. Symbols: ○, no DAP; ●, 1 μg of DAP per ml; ◑, 5 μg of DAP per ml; △, 10 μg of DAP per ml; ▲, 20 μg of DAP per ml; ×, 100 μg of DAP per ml.*

countered which did not belong in one of the three groups. This serotype was DAP and 8-azaguanine-sensitive and lipase-negative.

Because DAP sensitivity was a useful criterion in grouping the leptospires, a limited study of the action of this analogue was undertaken. The inhibitory effect of DAP on a group 1 leptospire, serotype *pomona* Wickard, was immediate and no observable growth occurred for 6 to 8 days (Fig. 1). However, the growth of DAP-resistant cells became apparent at this time. The appearance of DAP-resistant cells in media which contained concentrations of DAP that were initially inhibitory occurred in all cultures of group 1 leptospires tested. The DAP-resistant cells of serotype *pomona* Wickard, which appeared after 8 days incubation in media containing 10 μg of DAP per ml, were compared to cells from the original DAP-sensitive culture as to analogue sensitivity. Cells from the original culture were again inhibited by 10 μg of DAP per ml, whereas DAP-resistant cells were not significantly inhibited by as much as 500 μg of the analogue per ml. It was next determined whether the development of DAP resistance represented a genotypic or a phenotypic change. DAP-resistant serotype *pomona* Wickard cells were transferred 10 times in media with and without DAP. These cells were then compared to each other and to cells from the original DAP-sensitive culture. DAP resistance was maintained in the absence of the analogue (*see* Fig. 2). Similar results were obtained with serotype *copenhageni* M 20. These data suggest a selection of a new genotype in the presence of DAP rather than a phenotypic change in the cell population. In addition, the DAP-resistant mutants of serotype *pomona* Wickard were tested for resistance to the analogue of guanine, 8-azaguanine. The development of resistance to DAP was not accompanied by an increased resistance to 8-azaguanine (Fig. 2). Serotype *pomona* Wickard was also streaked on a 1% agar medium (2), and three isolated colonies were obtained and tested separately for DAP sensitivity. All three isolates were initially sensitive to 10 μg of DAP per ml and, after 6 to 8 days of incubation, DAP-resistant cells emerged. Whether DAP acts as a mutagen or the leptospires manifest a certain degree of genetic variation is not known at this time. However, it is unlikely that group 1 leptospires shift to group 2, since group 1 leptospires (serotypes *pomona*, *malaya*, and *paidjan*) selected for DAP resistance still possessed lipase activity.

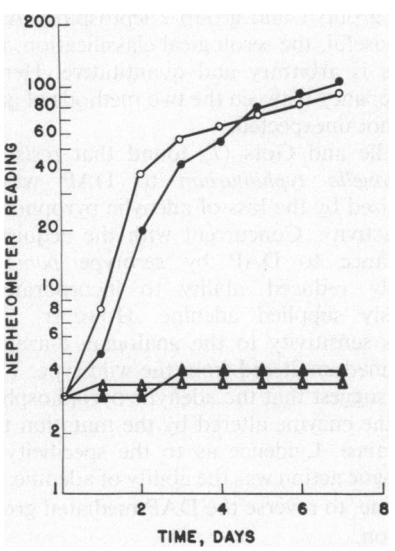

FIG. 2. *Growth response of serotype pomona Wickard DAP-resistant mutant to DAP and 8-azaguanine. Symbols: ○, no DAP; ●, 500 μg of DAP per ml; △, 200 μg of 8-azaguanine per ml; ▲, wild-type serotype pomona Wickard, 10 μg of DAP per ml. Inoculum size used in this experiment, 1% (v/v).*

TABLE 4. *Incorporation of C^{14}-8-adenine*

Serotypes tested	Counts per min per 10^8 cells[a]	Concn of DAP which inhibits growth
		μg/ml
pomona Wickard	1,001	5
pomona Wickard (DAP-resistant mutant)	260	>500
javanica SB 33	648	40
Sao-paulo Sao Paulo	570	>200

[a] Counts per min per 10^8 cells remained constant during the log and early stationary phases of growth. Results represent average incorporation of cells asayed after 3, 4, and 5 days of incubation at 30 C in the Tween 80-albumin medium containing 15 μg of ^{14}C-adenine per ml (specific activity: 3,760 counts per min per μg of adenine).

Male Syrian hamsters were infected with DAP-resistant cells derived from hamster-lethal cultures of serotypes *malaya* Mal 108 and *paidjan* Mal 1415 (group 1 leptospires) to determine whether acquisition of DAP resistance was related to loss of virulence. The DAP-resistant cells of these serotypes were lethal for hamsters and DAP-resistant cells were isolated from moribund animals.

The possibility that the development of resistance to DAP could be associated with a decreased incorporation of adenine was investigated. DAP-sensitive and DAP-resistant cells of serotype *pomona* Wickard were grown in the presence of 15 μg of ^{14}C-labeled adenine per ml. The development of resistance to DAP by serotype *pomona*

TABLE 5. *Reversal of DAP inhibition*[a]

Additions to rabbit serum medium	Increase in cell no. per ml[b]
None	40
10 μg of DAP per ml	1
10 μg of DAP per ml plus 10 μg of adenine per ml	31
10 μg of DAP per ml plus 0.1% yeast extract	38
10 μg of DAP per ml plus 10 μg of guanine per ml	1

[a] Test organism serotype pomona Wickard. Cultures incubated at 30 C for 5 days.

[b] All figures to be multiplied by 10^7.

was accompanied by a decreased incorporation of adenine (Table 4). The DAP-resistant mutant cells incorporated only 26% as much adenine as the original DAP-sensitive cells. ^{14}C-adenine incorporation by two naturally occurring DAP-resistant cultures was also examined. Although the DAP-resistant cells of serotypes *javanica* SB 33 and *sao-paulo* Sao Paulo incorporated less adenine than the DAP-sensitive serotype *pomona*, the difference was not as great as that observed between the two cultures of serotype *pomona* (Table 4).

The inhibitory action of DAP was readily antagonized by adenine in both the Tween 80-albumin medium and the rabbit serum medium. DAP loses 77% of its inhibitory activity in the presence of an equivalent amount of adenine, whereas the same concentration of guanine was without antagonizing activity (Table 5). Yeast extract contains adenine, and the presence of as little as 0.1% yeast extract in the test medium caused a 95% reversal of the growth inhibition mediated by 10 μg of DAP per ml (Table 5).

DISCUSSION

The genus *Leptospira* is presently represented by a single species, *L. interrogans*, and serological characteristics are the basis for the classification of these spirochetes (1). It was generally accepted that two major "complexes" existed within the genus: the "parasitic complex" and the "biflexa complex." Haapala et al. (*unpublished data*) have recently demonstrated that a considerable degree of genetic heterogeneity is present within the genus and that at least four distinct groups of leptospires exist.

Through the use of two purine analogues, DAP and 8-azaguanine, and lipase (trioleinase) activity, a simple and rapid means of separating the genus *Leptospira* into three groups is provided. Group 1 leptospires (8-azaguanine- and DAP-sensitive, lipase-positive) and group 2 leptospires (8-azaguanine-sensitive, DAP-resistant, lipase-negative) comprise the "parasitic complex" of leptospires. In addition to differing in DAP sensitivity and lipase activity, Haapala et al. (*unpublished data*) found group 1 and 2 leptospires to differ genetically. Moreover, cross-infection experiments with these groups of leptospires also suggest they are different. Recovery from a leptospiral infection results in immunity against the infecting serotype. In addition to this serotype-specific immunity, immunity also exists against leptospires belonging to different serotypes (interserotype immunity). It is of interest that interserotype immunity existed among group 1 leptospires, e.g., serotypes *icterohaemorrhagiae*, *canicola*, *pomona*, and *grippotyphosa*, but did not extend to a group 2 leptospire, serotype *tarassovi* (9). Group 3 leptospires represent the "biflexa complex." These leptospires can grow in the presence of 200 μg of 8-azaguanine per ml (6) and at low temperatures (5), characteristics which separate them from groups 1 and 2 leptospires. Group 3 and group 2 leptospires are also resistant to DAP, but differ in their lipase activity. Group 3 leptospires are genetically different from groups 1 and 2 leptospires (Haapala et al., *unpublished results*). The biological grouping of the leptospires does not always conform to their serological grouping. Several serogroups, such as *Tarassovi*, *Ballum*, and *Javanica*, contained only group 2 leptospires. Other serogroups, e.g., *Hebdomadis*, *Icterohaemorrhagiae*, and *Autumnalis*, contained both group 1 and group 2 leptospires. Although very useful, the serological classification of leptospires is arbitrary and quantitative. Hence, the discrepancy between the two methods of grouping was not unexpected.

Kalle and Gots (7) found that resistance of *Salmonella typhimurium* to DAP was characterized by the loss of adenylic pyrophosphorylase activity. Concurrent with the acquisition of resistance to DAP by serotype *pomona* was greatly reduced ability to incorporate exogenously supplied adenine. However, the mutant's sensitivity to the analogue, 8-azaguanine, remained unaltered from the wild type. These results suggest that the adenylic pyrophosphorylase was the enzyme altered by the mutation to DAP resistance. Evidence as to the specificity of the analogue action was the ability of adenine, but not guanine, to reverse the DAP-mediated growth inhibition.

The basis for the emergence of DAP-resistant cells from an analogue-sensitive culture is not understood at this time. The emergence of DAP-resistant cells from cloned cultures indicates that the parent culture was not simply a mixture of two

stable cell types. It was not determined whether the change from the DAP-sensitive to the DAP-resistant form was due to the presence of the analogue or occurred spontaneously in its absence. The three group 1 serotypes tested retained their lipase activity when they became DAP-resistant. Since none of the 74 leptospire cultures tested possessed the characteristics of these mutants (DAP-resistant, 8-azaguanine-sensitive, lipase-positive), this genetic variant is rarely selected for in nature or under the usual methods of cultivation of these leptospires. Serotypes *paidjan* and *malaya* did not lose their lethality for hamsters with the acquisition of resistance to DAP, suggesting that a major change in the genotype of these leptospires did not occur.

One leptospire serotype was encountered whose purine analogue sensitivity and lipase activity were unlike those of group 1, 2, or 3 leptospires. Serotype *kabura* was sensitive to both purine analogues but did not possess detectable lipase activity. This leptospire may represent a fourth group.

It was known for a number of years that the genus *Leptospira* contained both lipase-positive and lipase-negative serotypes (8, 10, 11, 13). However, since lipase production could not be correlated with other characteristics of the leptospires, it was not used as a differential criterion. The results of this investigation indicate that lipase activity is a useful differential criterion. The leptospires which do not have detectable lipase (trioleinase) activity are resistant to DAP but not to 8-azaguanine. In addition, they differ genetically from the lipase-positive leptospires (Haapala et al., *unpublished data*). Lipase production appears to be a stable characteristic of the leptospires. DAP-resistant mutants maintained their lipase activity as did leptospiral strains whose antigenic content was altered by passage in immune serum (13). Although both group 1 and group 3 leptospires produce lipase, Kmety et al. (12) demonstrated that their lipases differ from one another. They found that an antilipase serum prepared against a group 1 leptospire inhibited the lipase activity of all group 1 leptospires but not that of group 3 leptospires. The converse was observed with antilipase serum prepared from a group 3 leptospire.

Triolein was selected as the substrate for determining leptospiral lipase activity for the following reasons. (i) Triglycerides of short-chain fatty acids such as tributyrin are relatively nonspecific substrates and are slowly hydrolyzed by esterases (15) which leptospires are known to produce (4). (ii) Triolein represents the type of triglyceride present in the mammalian host and is a relatively specific lipase substrate.

The rabbit serum medium and the Tween 80-albumin medium were both satisfactory for conducting the DAP-sensitivity test. The test medium cannot be enriched with yeast extract, because it contains adenine which readily antagonized the action of DAP. Cultures tested for lipase activity were grown in the Tween 80-albumin medium, because the components of this medium were without detectable lipase activity. Rabbit serum medium was not satisfactory for this purpose, because the rabbit serum possessed lipase activity which interfered with the interpretation of the lipase assay results.

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