HAEMOLYSIS AS A MEANS OF DISTINGUISHING BETWEEN *LEPTOSPIRA INTERROGANS* SEROVARS *BALCANICA* AND *HARDJO*

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LEPTOSPIRES of the Hebdomadis serogroup have been isolated from a wide range of vertebrate hosts throughout the world (Communicable Diseases Center, 1966 and 1973). Only two serovars, *hardjo* and *balcanica*, belonging to this serogroup are known to be present in New Zealand.

Hardjo appears to be maintained by cattle (Johnson, Allan and Dennett, 1974; Ellis and Michna, 1977; Hellstrom, 1978), whereas *balcanica* has a wider host spectrum. *Balcanica* is endemic in possums (*Trichosurus vulpecula*) in New Zealand (Hathaway, Blackmore and Marshall, 1978) and in Australia (Durfee and Presidente, 1977); the original strain, 1627 Burgas, was isolated from a patient in Bulgaria (Babudieri and Mateew, 1961; Manev and Siromashkova, 1970), and subsequent strains were isolated from cattle and pigs in the Soviet Union (Semenova, Soloshenko and Ananyin, 1965).

Balcanica and *hardjo* are antigenically similar and the infections that they produce are not easily differentiated serologically (Hellstrom, 1978; Hathaway and Marshall, 1979). At present, the usual means of differentiating *balcanica* and *hardjo* is agglutinin absorption, which is not a routine procedure in most diagnostic laboratories.

Several serovars of various serogroups are known to be haemolytic; haemolytic activity has not been found in any serovar of the Hebdomadis serogroup (Russell, 1956; Kmety and Bakoss, 1961; Kasárov, 1970; Hodges, 1974) except serovar *haemolyticus*, strain Marsh (Alexander *et al.*, 1956). This report describes the in-vitro haemolytic activity of *balcanica* for human, bovine, ovine and possum red blood cells (RBC). This activity may be used as the basis of a simple test for the preliminary differentiation of *balcanica* and *hardjo* isolates.

MATERIALS AND METHODS

Organisms. A total of 14 *balcanica* and five *hardjo* strains were tested for haemolytic activity. Except for one *hardjo* isolate from a human (strain a), all *hardjo* strains had recently been isolated from cattle; the *balcanica* isolates were all from possums. Strains were maintained by weekly subculture in liquid EMJH Medium (Difco, Detroit, Michigan, USA). Periodic examinations were made for bacterial contamination. The identities of seven of the *balcanica* and three of the *hardjo* strains were subsequently confirmed by agglutinin-absorption tests at the WHO Reference Laboratory, Atlanta, Ga, USA. A *pomona* isolate from a pig was used as a positive control and a strain of *ballum*—a non-haemolytic serovar (Russell, 1956)—was used as a negative control. All cultures were aged 7 days and contained at least 10^7 organisms per ml.

Blood-agar plates. Red blood cells (RBC) from humans, cattle, sheep and possums were washed three times in sterile phosphate-buffered saline (0.85%). A serum sample from each donor was tested by the microscopic agglutination test (Cole, Sulzer and Pursell, 1973) against live *balcanica*, *ballum*, *copenhageni*, *hardjo*, *pomona* and *tarassovi*; the minimum serum dilution was always 1 in 24. Each plate (9 cm diameter) contained 10 ml of a 5% (v/v) suspension of RBC in Columbia Agar Base (BBL) over 10 ml of Agar Base (Difco) with 0.5% NaCl added. Other

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plates were similar but contained unwashed RBC. Ten wells were made in each plate by means of a sterile 8-mm cork borer. Plates were used within 3 weeks of preparation.

Plate haemolytic test. Each well was filled with 0.75 μ l of leptospire culture by means of a graduated automatic pipette fitted with a sterile disposable tip. In each plate one well was filled with 0.75 μ l of sterile medium. Plates were incubated at 30°C for 72 h in sealed containers. Zones of haemolysis were measured to the nearest 0.1 mm by means of a measuring magnifier. The measurement was taken from the edge of the well to the edge of the zone of complete haemolysis. Small outer zones of incomplete haemolysis were ignored.

Tube haemolytic test. Seven-day cultures (5 ml) in EMJH medium were rendered isotonic by the addition of 0.42 ml of 10% NaCl. One ml of each isotonic culture and 1 ml of 5% (v/v) washed human RBC were incubated at 37°C for 18 h in a sterile glass tube. An osmotic haemolysate (1.0 ml of 5% RBC suspension and 1.0 ml of distilled water) and a culture-medium control (1.0 ml of a 5% RBC suspension and 1.0 ml of isotonic medium) were incubated with each series of strains tested. After incubation all suspensions were centrifuged; each supernate was diluted with an equal volume of 0.85% saline, and its optical density read at 540 nm by means of a spectrophotometer (Bausch Lomb, Spectronic 20).

RESULTS

The table shows the ability of four strains of *balcanica* and one positive control (*pomona*) to haemolyse washed and unwashed RBC from two donors of each of the following species: man, ox, sheep and possum. Two strains of *hardjo* and one negative control (*ballum*) were negative; culture-medium controls were completely negative on unwashed RBC and mainly though not completely negative on washed RBC. In a second experiment the mean haemolytic activity of 14 strains of *balcanica* and five of *hardjo* was tested on washed and unwashed RBC from five human and five bovine donors. The mean measurements of the zones of haemolysis (in mm, $\pm$ SD) were as follows: *balcanica* on washed human and bovine RBC, 1.14 ± 0.27 and 1.09 ± 0.28 respectively; *balcanica* on unwashed human and bovine RBC, 0.82 ± 0.33 and 0.11 ± 0.29 respectively; *hardjo* negative on all RBC preparations except for a slight reaction (0.06 ± 0.03) on washed bovine RBC. The results for the positive and negative controls (*pomona* and *ballum* respectively) were exactly as in the first experiment (see table).

In all, three of six culture-medium controls gave a zone of incomplete haemolysis on washed RBC, and one gave a zone of complete haemolysis; no such reactions occurred on unwashed RBC. RBC from cattle, sheep and possums were much more susceptible in the washed state than in the unwashed state to haemolysis by *balcanica*. The greater resistance of unwashed RBC to haemolysis appeared to be associated with antibody to the Hebdomadis serogroup in the sera of donors. However, unwashed RBC from some seronegative donors were partially resistant to haemolysis.

In a tube test, haemolysis of washed human RBC from three donors, as measured by mean optical density, was tested in respect of six strains of *balcanica*, five of *hardjo*, one of *ballum*, a culture-medium control, and an osmotic haemolysate (100% haemolysis). The results, $\pm$ SD, were respectively 0.61 ± 0.12 , 0.11 ± 0.04 , 0.07 ± 0.03 , and 0.87 ± 0.03 . Reference to a standard curve showed that the percentage haemolysis values for *balcanica*, *hardjo*, and culture-medium controls were 70, 14 and 19, respectively.

Repeated testing showed that six strains of *balcanica* had lost, wholly or partially, their haemolytic activity for human RBC after 52 weekly subcultures.

DISCUSSION

Haemolytic activity has been demonstrated in serovars of the Canicola, Grippotyphosa, Autumnalis, Australis, Hebdomadis and Pomona serogroups (Alexander *et al.*, 1956; Russell, 1956; Kasárov, 1970). The haemolytic activity of serovar *balcanica* of the Hebdomadis serogroup has not previously been investigated.

All the *balcanica* strains studied were haemolytic, although some variation in activity was seen. Similar strain variation has been noted in other haemolytic serovars (Russell, 1956;

TABLE

Haemolysis of washed and unwashed red blood cells by serovars balcanica, hardjo, pomona and ballum, in a plate haemolytic test

RBC donor no.	Material tested for haemolysis	Leptospiral strain	Zone of haemolysis (mm) on							
			washed				unwashed			
			human RBC	bovine RBC	ovine RBC	possum RBC	human RBC	bovine RBC	ovine RBC	possum RBC
2	<i>Balcanica</i> culture	a	1.2	1.4	1.4	1.2	1.0	0.3	0.3	0
		b	1.0	1.1	1.3	1.1	0.8	0	0	0
		c	1.2	1.0	0.6	0.8	0.9	0	*0	0
		d	1.5	1.6	1.2	0.9	1.4	0	0	0
	<i>Hardjo</i> culture	a	0	0	0	0	0	0	0	0
		b	0	0	0	0	0	0	0	0
	<i>Pomona</i> culture	a	1.4	1.6	1.0	1.0	0.2	1.2	0.5	0
	<i>Ballum</i> culture	a	0	0	0	0	0	0	0	0
	Culture medium	...	0*	0	1.0	0*	0	0	0	0
	<i>Balcanica</i> culture	a	1.3	2.0	N	N	1.2	0	N	N
		e	0.9	1.4	N	N	0.7	0	N	N
		f	0.9	1.0	N	N	0.8	0	N	N
		g	0.9	0.9	N	N	0.7	0	N	N
	<i>Hardjo</i> culture	h	1.2	1.7	N	N	0.7	0	N	N
		i	1.0	1.5	N	N	0.8	0	N	N
		a	0	0	N	N	0	0	N	N
		c	0	0	N	N	0	0	N	N
	Culture medium	c	0	0	N	N	0	0	N	N
			0*	0	N	N	0	0	N	N

N = not done.

* Zone of incomplete haemolysis.

Kmety and Bakoss, 1961; Chorvath, 1975). All strains used in the present study had been recently isolated; serial subculturing for more than a year sometimes resulted in a partial or complete loss of haemolytic activity. The use of laboratory strains by the earlier workers referred to above may have contributed to the observed variation in haemolytic activity.

Hardjo cultures were non-haemolytic on blood-agar plates, and in the tube test the degree of haemolysis of washed human RBC produced by such cultures was less than that produced by sterile culture medium. Hodges (1974) similarly found New Zealand *hardjo* strains to be non-haemolytic. The inability of *hardjo* to haemolyse human and bovine RBC may serve as the basis for a simple in-vitro test for the differentiation of *hardjo* and *balcanica*. The most consistent results were obtained with washed human RBC and the maximum haemolytic activity of cultures was found to occur at the peak of leptospiral growth. The test was not significantly affected by blood group.

A haemolytic effect, usually incomplete, was produced on washed RBC by sterile culture medium. This reaction was completely inhibited by the presence of leptospires in the culture medium; *hardjo* and *ballum* cultures were non-haemolytic on plates on which culture-medium reactions were seen. No explanation is readily apparent. There were no significant differences in the osmolarity of culture medium that had supported the growth of 7-day *hardjo* and *ballum* cultures and that of the sterile control medium.

Infection with leptospires of the Hebdomadis group is endemic in cattle, sheep and possums in New Zealand. The experiments showed that serum antibody was associated with the resistance of the unwashed RBC of some individuals to haemolysis by *balcanica*. The ability of serum agglutinins to inhibit the haemolytic activity of leptospires has been reported by others (Russell, 1956; Kemenes, 1974). It has also been demonstrated *in vitro* that haemolysin-inhibit-

ing antibodies will cross-react with heterologous haemolysins (Alexander *et al.*, 1971; Hodges, 1974).

The differentiation of *balcanica* and *hardjo* by their action on human RBC cannot be regarded as definitive. Nevertheless it may be useful in screening large numbers of *Hebdomadis* serogroup isolates in countries such as New Zealand, where experience has shown that *Hebdomadis* serogroup isolates are likely to be either *hardjo* or *balcanica*.

SUMMARY

Leptospira interrogans serovar *balcanica* was haemolytic for washed human, cattle, sheep and possum red blood cells (RBC); serovar *hardjo* was non-haemolytic. A test for haemolysis may prove useful in the preliminary differentiation of *balcanica* and *hardjo* isolates. *Balcanica* was non-haemolytic for unwashed RBC from human and bovine donors whose serum contained *Hebdomadis* serogroup antibody. Haemolytic activity tended to disappear with repeated subculture of *balcanica*.

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