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MICROBIOLOGY

In vitro Agglutination of Bovine Erythrocytes infected with *Babesia argentina*

THE presence of antibodies capable of agglutinating intracellular schizonts of *Plasmodium knowlesi* has been reported[1] and a parasite agglutination test described[1–2]. This communication reports a similar test for *Babesia argentina* using heavily parasitized bovine erythrocytes.

Splenectomized calves, 3–6 months old, were infected with *B. argentina* by intravenous inoculation of blood from carrier cattle. When 2 per cent of the erythrocytes in jugular blood were infected, blood was collected in bottles containing EDTA (disodium salt of ethylenediamine tetraacetic acid) as anticoagulant. If the peak parasitaemia was below this level, blood was transferred to another splenectomized calf and the procedure repeated until the 2 per cent parasitaemia was obtained.

One per cent erythrocyte suspensions in physiological saline were prepared which contained approximately 80 per cent of their cells infected with *B. argentina*. The infected cells were first concentrated from parasitized blood by a method described by Mahoney[3]. Appropriate control suspensions were also prepared from normal blood taken from each calf before infection. These suspensions of infected and uninfected cells were used as antigens for the agglutination test.

The agglutination test was conducted in 'Perspex' haemagglutination trays. Sera were diluted 1/5 in physiological saline containing 1 per cent normal calf serum and heated at 56° C for 30 min. Doubling dilutions of serum were prepared and to 0·2 ml. of each dilution an equal volume of the infected cell suspension was added. A control test using the uninfected cell suspension was included with each serum. The plates were shaken and allowed to stand at room temperature for 3 h.

Reactions were read macroscopically and microscopically (× 100), the latter after resuspending by gentle shaking. Macroscopic reactions were graded as follows: + + + +, a mat of cells completely covering the bottom of the cavity without any ring of cells around its periphery. The surface had a granular appearance and the edges of the mat were sometimes folded; + + +, similar to the + + + + reaction but with a thin ring of cells around the periphery; + +, a distinct ring of cells with only a slight granular appearance; +, a thick ring of cells with a diameter approximately half that of the mat in the + + + + reaction; ± or −, a discrete button or thick ring of cells was present.

The titre of the serum was taken as the highest dilution showing a + + reaction macroscopically.

Microscopically the amount of agglutination was estimated at the 1/5 dilution as follows: + + + +, many clumps of over 200 cells; + + +, many clumps of over 50 cells; + +, less numerous clumps of over 20 cells; +, odd clumps of less than 20 cells.

Parasites from five isolations of *B. argentina* from geographically separated natural outbreaks in New South Wales were used to prepare suspensions. Isolates were maintained in individual carriers at the laboratory and designated with the name of the locality from which they came (Table 1). A sixth isolate (Gayndah) was obtained from Dr D. F. Mahoney, CSIRO, Yeerongpilly. Occasionally before inoculation, parasites were preserved by freezing infected whole blood to −79° C and storing at this temperature.

Antisera were collected from the same six carrier animals used to provide isolates for suspensions but were taken 2 to 6 weeks later. They were stored at −20° C without preservative.

The results of testing these suspensions with homologous and heterologous antisera are shown in Table 1. With each suspension the highest titre and greatest degree of clumping was obtained with the homologous antiserum. Reactors with the heterologous antisera were slight.

Preliminary studies on the specificity of the reaction have been undertaken. Of 450 non-infected animals tested with the Gayndah isolate suspension only four (0·9 per cent) showed any agglutination at the 1/10 dilution and only one (0·2 per cent) at the 1/20 dilution. The greatest amount of clumping recorded was + + at the 1/5 dilution with one serum. Similar results were obtained with 100 of these sera when tested with the other five isolate suspensions.

The reaction appeared to be highly specific for each isolate. The isolates used were possibly antigenic variants of one or more strains of *B. argentina* arising by a process similar to that demonstrated by the agglutination test in chronic *P. knowlesi* infections[2]. The test described here is at present being used to investigate the possibility of similar antigenic variation in *B. argentina* infections. If basic strain antigens can be identified and strains of the parasite classified, the technique would be valuable in epidemiological studies.

The effect of these agglutinating antibodies on immunity also needs to be investigated. Agglutinins in *P. knowlesi* were considered to be important in terminating relapses, but another immune response was also thought to be present and partially able to inhibit relapses[2]. A similar state of affairs may exist with *B. argentina* infections.

Table 1. AGGLUTINATION TEST RESULTS OF SERA FROM SIX CATTLE INFECTED WITH SEPARATE ISOLATES OF *Babesia argentina* AGAINST ANTIGENS FROM THE SAME ANIMALS

		Antigen					
	Isolate	Gayndah	Cougal	Piggabeen	Dalguigan	Wiangaree	Roseberry
Serum	Gayndah	*1,250 (+ + + +)†	0 (−)	0 (−)	40 (+ +)	0 (−)	0 (−)
	Cougal	160 (+ +)	5,000 (+ + + +)	40 (+)	20 (+)	10 (+)	40 (+)
	Piggabeen	0 (−)	0 (−)	640 (+ + + +)	10 (+)	5 (+ +)	5 (+)
	Dalguigan	20 (+ +)	—	—	1,250 (+ + +)	0 (−)	40 (+ +)
	Wiangaree	0 (−)	0 (−)	0 (−)	0 (−)	320 (+ + + +)	0 (−)
	Roseberry	10 (+)	20 (+ +)	0 (−)	20 (+)	0 (−)	5,000 (+ + + +)

* Reciprocals of agglutination test titres.
† Size and number of agglutinated clumps at the 1/5 serum dilution.

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It would seem that the development of an effective vaccine able to produce a sterile immunity for *B. argentina* would depend on the importance of these antigens and their variability.

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CYTOLOGY

Growth-promoting Effect of Lysolecithin on Chinese Hamster Cells *in vitro*

WHILE the importance of amino-acids[1,2], salts[3] and vitamins[4] for growth of mammalian cells in culture is well established, the role of lipids remains uncertain. With the exception of free fatty acids[5], no naturally occurring lipids such as glycerides, phospholipids or cholesterol have been shown to promote growth of mammalian cells *in vitro*.

Lysolecithin was until recently considered to be a toxic haemolysing substance generated in the blood by the action of snake venom. In 1960, however, Lands[6] showed that lysolecithin was an intermediate in the synthesis of lecithin in rat liver homogenates. Further, lysolecithin was found in both rat[7] and man[8] to be a normal constituent of plasma and to be associated exclusively with the albumin fraction[9]. Stein and Stein[10,11] then showed that albumin-bound lysolecithin was incorporated by intact cells and utilized in the synthesis of lecithin.

I have found that albumin-bound lysolecithin has a definite growth-promoting effect on Chinese hamster cells in culture. The effect seems to be caused by the uptake of intact lysolecithin and not by breakdown products.

The lysolecithin (Koch–Light Laboratories, Ltd.), a 1-acyl phosphatidylcholine prepared from egg lecithin by degradation with snake venom phospholipase, was shown to be pure by thin layer chromatography. Analysis by gas–liquid chromatography showed that 94 per cent of the fatty acid moiety was saturated, the principal components being palmitic acid (71 per cent) and stearic acid (21 per cent). The unsaturated acids (6 per cent) were chiefly oleic (2 per cent) and linoleic acid (2 per cent). Bovine plasma albumin (Armour, Fraction V) was extracted by the method of Goodman[12] and a lysolecithin–albumin complex was prepared and purified by the method of Stein and Stein[13]. The lysolecithin content of the albumin was determined by thin layer chromatography of the albumin phospholipids followed by phosphorus analysis[14].

Chinese hamster cells, line *CHEF*, were routinely grown in the Dulbecco–Vogt modification of Eagle's medium[15] supplemented with 20 per cent calf serum. Experimental cultures were inoculated with 8×10^4 cells in each Petri dish (diameter 32 mm) in 1·5 ml. of medium and allowed to attach. After 4 h the medium was removed and the experimental medium added. The plates were then incubated for 3 days. Cell numbers were determined at the beginning and at the end of this period by counting the cells within five squares etched into the bottom of the plates.

The experimental medium was identical with the usual medium except that it was free of serum and contained 1 per cent extracted albumin with and without added lysolecithin. The final concentration of lysolecithin was 75 µmoles/l. of medium, and the molar ratio of lysolecithin : albumin was 1 : 2 (assumed molecular weight of albumin 67,000). Without addition the concentration of lysolecithin in the medium was less than 1 µmole/l.

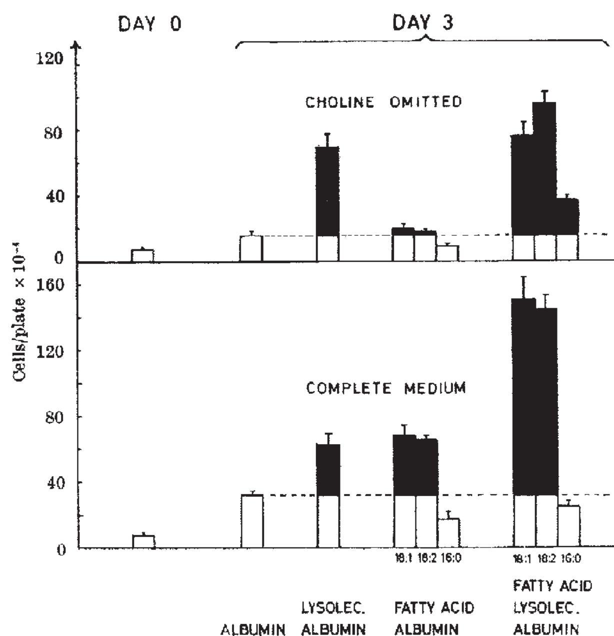

Fig. 1. Effect of albumin-bound lysolecithin and fatty acids on growth of Chinese hamster cells *in vitro*. The columns represent the average of four experiments each including eighteen plates, the vertical bars being the standard error of the mean. The black portion of the columns indicates the increase in cell number due to the presence of albumin-bound lipid. Oleic (18 : 1), linoleic (18 : 2) and palmitic (16 : 0) acids are designated by the number of carbon atoms and double bonds in the molecule[18].

The addition of lysolecithin to the medium (Fig. 1) increased growth significantly ($P < 0·01$). This effect was evident both in the presence and absence of choline, but the effect was greater when choline was omitted from the medium.

Fatty acids have been shown to promote growth of cells in culture[5] and some cells have lysolecithinases (acyl hydrolases) associated with their surfaces[11,16], so the question arose as to whether the growth-promoting effect was caused by free fatty acids liberated by the action of lipase or to the uptake of lysolecithin as such. To examine this question individual high purity fatty acids (Applied Science Laboratories, Inc.) were tried as substitutes for lysolecithin. The fatty acids were combined with extracted albumin and their concentration determined by titration[17]. The final medium contained 75 µmoles of free fatty acids/l., while without additions the concentration was less than 3 µmoles/l.

In the presence of choline, the unsaturated acids, oleic and linoleic, stimulated growth markedly, whereas the saturated palmitic acid inhibited growth (Fig. 1). About 94 per cent of the fatty acids of lysolecithin were saturated, and more than 70 per cent was palmitic acid, so that it seems unlikely that the observed effect of lysolecithin could be caused by its fatty acid moiety in the form of free fatty acid. Furthermore, the maximal concentration of unsaturated free fatty acids attainable, if all the lysolecithin were hydrolysed, was found to be too low to promote growth (my unpublished results).

Lysolecithin has been found to be an intermediary in the synthesis of lecithin in other cells, and so these results suggest that lysolecithin stimulates growth by promoting synthesis of lecithin. The following observation seems to support this hypothesis. Equimolar amounts of lysolecithin together with oleic or linoleic acid, in the complete medium, stimulated growth to a degree that exceeded the sum of their individual effects ($P < 0·01$ by paired comparison) (Fig. 1). This synergism suggests that lysolecithin and unsaturated fatty acids have a common mechanism

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