THE BEHAVIOUR OF *BRUCELLA ABORTUS* WITHIN MACROPHAGES SEPARATED FROM THE BLOOD OF NORMAL AND IMMUNE CATTLE BY ADHERENCE TO GLASS

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INVESTIGATIONS of the intracellular behaviour of *Brucella abortus* have figured prominently in studies of cellular immunity (Elberg, 1960; Jenkin and Rowley, 1963; Mackaness, 1964; Cohn and Hirsch, 1965). In most of these studies artificially produced monocyte populations from normal and immune laboratory animals were used (Holland and Pickett, 1958; Stinebring, Braun and Pomalès-Lebron, 1960). Macrae and Smith (1964) made the first studies with the cells of a naturally susceptible host; they could not detect significant differences in the behaviour of *Br. abortus* within the phagocytes from normal and immune cattle. However, they used a test developed by Smith and FitzGeorge (1964*a*) for studies of the chemical basis of intracellular growth in which *Br. abortus* was phagocytozed by cells of bovine " buffy coat ". Although some phagocytosis by mononuclear cells occurred, in the main, this test measured the behaviour of *Br. abortus* within the polymorphonuclear cells because these predominated in the " buffy coat " and have a greater ability than monocytes both for ingesting *Br. abortus* and inhibiting its subsequent intracellular growth (R. B. FitzGeorge, P. W. Harris-Smith and H. Smith—unpublished observations). Hence, in view of the previous work with monocytes from laboratory animals, the possibility remained that differences between the behaviour of *Br. abortus* within cells of immune and non-immune cattle could be detected if the less destructive monocytes were used for the intracellular studies. To investigate this possibility, a method was required for separating mononuclear phagocytes from the other cells of bovine " buffy coat " in sufficient quantity and of adequate quality for subsequent intracellular studies. This paper describes first the establishment of such a method and second the behaviour of *Br. abortus* in monocytes separated from the blood of normal and immune cattle.

MATERIALS AND METHODS

Separation of macrophages from calf blood

Defibrinated calf blood (1 l.) was centrifuged (1200 **g**.) for 15 min. The serum was removed and reserved for subsequent use in the test (see below). The " buffy coat " (*ca* 50 ml. $\equiv 5 \times 10^9$ cells) was collected, placed in a Roux bottle in an atmosphere of 5 per cent CO_2/air, and left for 12 hr. at 37°. Cells not adhering to glass were removed by exhaustive washing (*ca* 10 times with 20 ml.) with a mixture (1 : 1) of calf serum and saline. Fresh calf serum (50 ml.) was added to the mixture of macrophages and polymorphonuclear cells which

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had adhered to the glass and incubation at 37° was continued for 10–12 days. During this time the polymorphonuclear cells died and became detached from the glass. The Figures show photographs of the cells adhering at 1, 3, 5 and 10 days. After 10–12 days the medium was removed and replaced by a solution (50 ml.) of trypsin (crystalline, Armour Pharmaceutical Co. Ltd., 0·1 per cent w/v) in phosphate buffer (I 0·26, pH 8·0). After 10–30 min. at 37° the macrophages became detached; they were collected by centrifugation (750 g.; 10 min.) and resuspended in fresh serum (*ca* 50 ml.; 1 × 10^5 per ml.). Amounts (1 ml.) of the cell suspension were placed in Leighton tubes which were incubated at 37° in an atmosphere of 5 per cent CO_2/air. About half of the macrophages reattached themselves to the glass and were then ready for parasitization.

This process using one Roux bottle and then distributing the final suspension into Leighton tubes was that finally adopted after much preliminary quantitive work described below.

Estimation by a direct microscopical count of the concentration of macrophages attached to Leighton tubes.—A binocular microscope (× 8 eyepiece and × 10 objective) was used. An area equivalent to 0·6 sq. mm. of an eye-piece graticule was calibrated by reference to a stage micrometer. The number of adhering macrophages on 9 separate areas (0·6 sq. mm.) of an upturned Leighton tube (flat area, 480 sq. mm.) was counted (approx. 1000 cells in all) and from the average count the total number of cells in the Leighton tube was calculated. Table I shows the comparison between cell-counts obtained by this method and those obtained in Neubauer chamber-counts of the suspension of cells.

TABLE I.—*The Macrophage Content of Leighton Tubes Estimated both by Direct Examination and by Neubauer Chamber Counts*

	Tube	Direct count (× 10^4)	Count in Neubauer chamber (× 10^4)	Neubauer count / Direct count
Test 1	1	6·7	10	1·5
	2	9·0	12	1·3
	3	7·4	8·6	1·2
	4	8·4	11	1·3
Test 2	1	5·8	6·7	1·2
	2	6·6	6·5	1·0
	3	6·2	7·9	1·3
	4	5·8	7·5	1·3
	5	6·2	7·8	1·3
	6	6·0	8·1	1·3

Methods—see text.

Statistical analysis of the counts showed that the counts made on individual tubes by each method were consistent (the direct counts being the better); therefore the ratios of the counts shown in the last column are real and consistent.

The behaviour of the cells of " buffy coat " which adhere to glass; survival and increase in numbers of the macrophages.—Suspensions of aliquots (1 ml.) of the " buffy coat " were placed in Leighton tubes and treated as described above for the suspension in the Roux bottle. The cells which attached to the flat glass surface were observed each day for a period of 26 days. Initially the adhering cell population was mainly polymorphonuclear cells and macrophages (see Figs.), however after the 5th day only macrophages could be seen (see Figs.), the remainder of the cells having died or at least lost their power of adhesion to the glass surface. On the 6th day, the macrophage population was sufficiently pure for reliable population counts to be made. These counts showed: (1) a variation from tube to tube (often 10-fold) in the number of adhering macrophages despite the fact that all received initially the same volume of " buffy coat "; (2) a slow increase (approx. 7-fold over the count at 6 days) in the number of macrophages for 12–15 days after which time the population remained fairly constant at least until 26 days (Table II). There was no obvious contamination with other cells (*e.g.* fibroblasts) and mitosis was seen. Hence, these bovine macrophages appeared to multiply slightly during the first 12–15 days, a fact already known in relation to macrophages of other species (Jacoby, 1965). Even after 26 days on glass the macrophages appeared perfectly healthy; they excluded eosin and had the ability to phagocytoze.

TABLE II.—*The Increase in Number of Bovine Macrophages during Incubation on a Glass Surface*

Tube	Counts ($\times 10^4$) of macrophages after incubation for the following number of days: 6	8	12	15	23	26
1	1·6	2·9	7	10	14	14
2	1·3	1·9	3·5	4·8	5·8	8·5
3	1·3	2·1	5·3	8·5	10	14
4	1·2	2·6	8·1	11·1	10	12
5	1·6	1·3	8	11·1	15	18
6	1·4	2·6	5·5	6·9	9·1	12
Mean	1·4	2·2	6·2	8·7	10·6	13·1

For this experiment 6 Leighton tubes were chosen from many (50–60) for evidence of uniformity of macrophage content at day 6.

This experiment is representative of 4 similar experiments.

Removal of macrophages from glass by treatment with trypsin and their re-attachment to fresh glass surfaces for subsequent parasitization.—For subsequent work on the intracellular behaviour of *Br. abortus* (see below) Leighton tubes containing the same number of bovine macrophages were required. Uniform populations of macrophages in different tubes could not be obtained directly by distributing the same volume of " buffy coat " into the tubes and incubating for 10–12 days (see above). Hence the initial separation of macrophages was conducted in bulk in a Roux bottle (see above) and after 10–12 days incubation, the macrophages were resuspended by treatment with trypsin so that equal numbers (*ca* 1×10^5) could be distributed into Leighton tubes for subsequent work. Preliminary experiments showed that treatment with trypsin: (1) did not lyse the macrophages since all counts of suspensions were the same as those indicated by direct counts on the original surface layers; (2) did not affect their dye-exclusion properties; and (3) did not prevent subsequent attachment of the macrophages to fresh glass surfaces. Approximately half of the macrophages in the suspension re-attached themselves; and the changes in counts of these re-attached macrophages which occurred after further incubation were similar to those described in Table II for macrophages attached directly from the buffy coat.

Parasitization of macrophages with Br. abortus *and measurement of intracellular growth*

Leighton tubes containing the same number of macrophages (*ca* 5×10^4) and prepared as described above were incubated at 37° for 1–2 days to allow the macrophages to stabilize after treatment with trypsin. The number of macrophages on the glass surface of the Leighton tubes was counted directly. In numerous routine tests these direct counts were the same (within the factor indicated in Table I) as those obtained later in the test (see below) by Neubauer chamber counts on suspensions of macrophages released from the glass surface by treatment with trypsin.

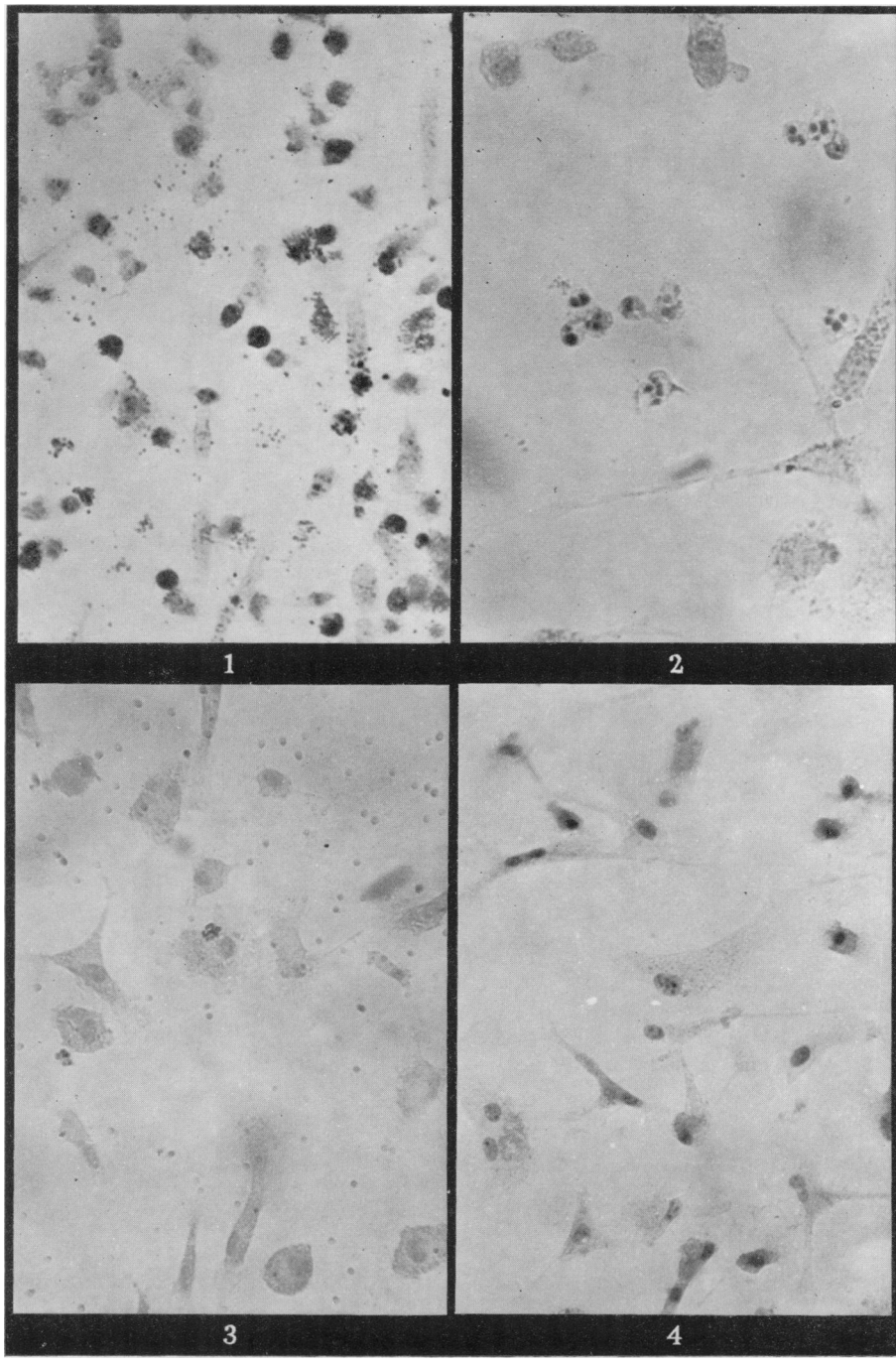
Br. abortus (strain 544) was grown on a slope of tryptic digest meat agar for 18 hr. at 37° in 10 per cent CO_2/air and suspended in saline containing 10 per cent tryptic meat broth. The suspension was counted in a Helber chamber and diluted in heated (56°, 1 hr.) calf serum (containing 1 per cent unheated calf serum to promote phagocytosis) until 1 ml. contained 100 *Br. abortus* for each macrophage in the Leighton tubes.

Phagocytosis of *Br. abortus* by the macrophages in the Leighton tubes was obtained by replacing the medium in the latter by the suspension of *Br. abortus* and incubating at 37° for

EXPLANATION OF PLATE

Cells of calf-blood buffy coat adhering to glass after incubation (37°) in isologous serum ($\times 120$):

FIG. 1.—One day.
FIG. 2.—Three days.
Fig. 3.—Five days.
FIG. 4.—Ten days.



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1 hr. A small but adequate phagocytosis occurred. Extracellular organisms were then killed by replacing the non-bactericidal heated serum with fresh calf serum containing streptomycin (10 μg/ml.) and incubating at 37° for 1 hr.

The total number of macrophages and intracellular *Br. abortus* in each of 4 Leighton tubes was determined at this stage and after a further incubation at 37° for 40 hr. by the following procedure. The bactericidal mixture was replaced with 0·25 ml. of a trypsin solution in phosphate buffer (see above) and the tube was rocked mechanically at 37° until the cells became detached (10–30 min.). After cooling to 0°, 1 drop (0·025 ml.) was taken for estimating the number of suspended macrophages; it was added to 1 drop of 10 per cent formol/saline in a Dreyer tube and the number of macrophages was counted in a Neubauer chamber (cells in 9 cu. mm. counted). Saline (0·75 ml.) was then added to the remainder of the macrophages which were then collected by centrifugation (250 **g**; 7 min.) and suspended in distilled water (1 ml.) containing 0·002 per cent tryptose. Intracellular *Br. abortus* were liberated by shaking with glass beads in a bijou bottle for 5 min. and counted by plating the suitably diluted suspension on plates of tryptic digest meat agar containing 5 per cent peptic digest of sheep blood. Table III shows that at the beginning of the period of intracellular

TABLE III.—*Total Number of Macrophages and* Br. abortus *in Leighton Tubes (in quadruplicate) at the Beginning of the Period of Intracellular Growth*

	Tube	No. of macrophages ($\times 10^4$)	No. of *Br. abortus* ($\times 10^4$)
Test 1	1	10	3·1
	2	11	3·2
	3	10	2·9
	4	12	3·0
Test 2	1	5·2	1·5
	2	5·4	1·5
	3	5·5	1·6
	4	3·9	1·3
Test 3	1	3·0	1·4
	2	3·1	1·5
	3	3·1	1·2
	4	2·9	1·3
Test 4	1	3·5	2·3
	2	3·4	2·5
	3	4·0	2·4
	4	3·9	2·2

Methods—see text.

Statistical analysis: according to the probability level of all relevant χ^2 values, the 4 tubes returned consistent counts both for the numbers of macrophages and the numbers of *Br. abortus*.

The data are representative of several similar tests.

growth each of the quadruplicate Leighton tubes contained the same (within reasonable limits) number of macrophages adhering to the glass surface and the same (within reasonable limits) total number of phagocytozed *Br. abortus*. Table IV shows that during the 40 hr. period of intracellular growth (which follows the 10–12 day incubation of uninfected macrophages in the Roux bottle) the number of macrophages in the Leighton tubes increased (Table II) irrespective of whether they were parasitized with *Br. abortus* or not.

Comparison of the intracellular growth of Brucella abortus *in macrophages from normal and immune calves.*—To provide blood-macrophages from immune animals, a group of calves were given the following doses of live *Br. abortus* (strain 544) $1{\cdot}5 \times 10^{11}$, $1{\cdot}5 \times 10^{11}$, 3×10^{12}, 3×10^{12} at intervals of 2 months. The first dose was given partly i.v. and partly by the i.m. and i.p. routes, but thereafter only the latter routes were used. After an interval of 6 months and during the following 6 months, the macrophages and serum of these animals were compared in intracellular growth tests with those from similar normal animals. Their serum agglutinated *Br. abortus* in a dilution of $\frac{1}{1280}$ or more.

To compare the behaviour of *Br. abortus* within the macrophages of normal and immune calves, macrophages (and serum) were separated from blood taken from the animals on the same day. Isologous serum was used throughout at appropriate stages in the tests for

TABLE IV.—*The Numbers of Macrophages in Leighton Tubes (in quadruplicate) at the Beginning and at the End of the Period of Intracellular Growth of* Br. abortus

	Number of macrophages ($\times 10^4$) present: Initially	after 40 hr.	Average increase 0–40 hr. (per cent)
Infected macrophages			
Tube 1 .	3·0	3·5	36
2 .	3·2	4·2	
3 .	3·3	4·6	
4 .	3·4	4·5	
Normal macrophages			
Tube 1 .	2·9	4·4	33
2 .	3·0	3·4	
3 .	2·7	3·9	
4 .	3·4	4·1	

Methods—see text.

Statistical analysis of the counts showed consistent counts in each of the quadruplicate tubes and no significant difference between the average increase in count of the infected and normal macrophages. These figures are representative of several similar tests.

intracellular growth except in the experiments investigating the influence of immune serum on normal cells and *vice versa*. In these tests pooled normal serum (from the 3 normal animals) and immune serum (from the 3 immune animals) were used at the phagocytosis stage of the test and subsequently.

RESULTS

The slower multiplication of virulent Brucella abortus *within macrophages of immune calves compared with that in macrophages of normal animals*

The results in Table V show that *Br. abortus* does not multiply in the macrophages from immune calves nearly so extensively as in macrophages from normal animals. These experiments were conducted in the presence of isologous serum.

The absence of influence of immune serum on the multiplication of Br. abortus *within calf macrophages*

The results in Table VI show that the multiplication of *Br. abortus* in macrophages from normal and immune animals was the same whether pooled immune or pooled normal serum was used in the test for intracellular growth instead of isologous serum. Furthermore the changes of serum did not affect the essential result indicated by Table V, namely that multiplication within macrophages from immune animals was less than that in cells from normal calves.

DISCUSSION AND SUMMARY

Macrophages have been separated from the " buffy coat " of bovine blood by prolonged adhesion (12–15 days) on glass surfaces; many of the other cells present in " buffy coat " did not adhere and those that did so initially (mainly polymorphonuclear cells) soon died or became detached. Macrophages separated in this manner appeared to multiply slowly up to 12–15 days (cf. macrophages of other species, Jacoby, 1965) and then the population remained more or less constant but viable for at least 26 days. This process might be used to separate macrophages from the blood of other animal species for experiments on the mechanisms of intracellular growth and cellular immunity.

TABLE V.—*The Growth of* Br. abortus *in Macrophages from Normal and Immune Cattle in the Presence of Isologous Serum*

Origin of cells	Tube	No. of viable *Br. abortus* per macrophage: Test 1 0 (hr.)	Test 1 40 (hr.)	Test 1 mean at 40 hr. / mean at 0 hr.	Test 2 0 (hr.)	Test 2 40 (hr.)	Test 2 mean at 40 hr. / mean at 0 hr.
Normal calves:							
No. 222	1	0·33	24	64	0·17	28	190
	2	0·29	18		0·14	33	
	3	0·38	22		0·16	29	
	4	—	—		0·14	27	
No. 240	1	0·28	21	86	0·71	44	63
	2	0·26	18		0·67	59	
	3	0·25	21		1·52	58	
	4	0·15	21		0·71	68	
No. 101	1	1·06	136	120	0·51	146	255
	2	1·56	200		0·51	114	
	3	1·88	204		0·52	—	
Immune calves:							
No. 254	1	1·36	9·7	14	0·19	1·9	9
	2	0·55	8·8		0·21	1·9	
	3	0·48	9·4		0·18	1·7	
	4	0·56	13·3		0·18	1·6	
No. 246	1	0·90	12·9	15	0·65	17·7	29
	2	0·63	10·2		0·57	19·0	
	3	0·61	9·1		0·60	16·6	
	4	0·56	9·1		—	—	
No. 213	1	0·88	7·2	5	0·56	18·6	37
	2	1·07	5·3		0·67	19·6	
	3	1·12	4·2		0·57	29·7	
	4	1·03	5·2		0·64	22·0	

Methods—see text.

Statistical analysis of the counts showed that the increase in the number of *Br. abortus* in the normal macrophages was significantly greater than that in the immune cells.

Brucella abortus multiplied more slowly in the macrophages of immune calves than in those of normal animals. Immune serum had no effect on the multiplication of *Br. abortus* in the macrophages of normal or immune animals (Smith and FitzGeorge, 1964*a*, *b*; Blanden, Mackaness and Collins, 1966). It seems probable that immunity to brucellosis in calves depends more on cellular than on humoral mechanisms. It must be remembered however that significant differences in respect to intracellular growth of *Br. abortus* between the cells of these immune and normal animals have only been demonstrated for blood macrophages separated and possibly selected by the method described here. In earlier work relating to the intracellular survival and growth of *Br. abortus* mainly within polymorphonuclear cells, no significant differences between normal cells and immune cells could be demonstrated (Macrae and Smith, 1964); although again antiserum had no effect on intracellular events (Smith and FitzGeorge, 1964*b*). In the normal animal, polymorphonuclear cells are more destructive for *Br. abortus* than macrophages and it is possible that survival in the latter is a mechanism of infection in the normal animal which is reduced in the immune animal.

TABLE VI.—*The Growth of* Br. abortus *in Macrophages from Normal and Immune Cattle in the Presence of Either Normal or Immune Serum*

Origin of cells	Tube	No. of viable *Br. abortus* per macrophage: Test 1 (Pool normal serum) 0 (hr.)	40 (hr.)	mean at 40 hr. / mean at 0 hr.	Test 2 (Pool immune serum) 0 (hr.)	40 (hr.)	mean at 40 hr. / mean at 0 hr.
Normal calves:							
No. 222	1	0·26	12·0	52	0·21	9·5	48
	2	0·27	14·3		0·21	12·7	
	3	0·26	15·1		—	8·1	
No. 240	1	0·48	10·8	34	0·24	11·6	50
	2	0·26	12·7		0·22	11·2	
	3	0·26	10·6		0·15	7·8	
No. 101	1	0·54	107·0	106	0·81	84·0	108
	2	1·25	107·0		0·76	107·0	
	3	1·26	—		0·98	83·0	
Immune calves:							
No. 254	1	1·10	6·8	6	1·40	11·3	7
	2	1·10	7·7		1·50	9·2	
	3	1·05	6·3		1·20	9·1	
No. 246	1	0·76	16·0	23	0·67	14·6	23
	2	0·54	13·3		0·54	13·0	
No. 213	1	0·70	15·9	22	0·70	13·8	22
	2	0·70	17·7		0·70	13·9	
	3	0·70	14·6		0·60	13·6	
	4	0·80	16·0		0·50	12·6	

Methods—see text.

Statistical analysis of the counts showed that (a) the increase in brucella in normal macrophages was significantly higher than that in immune cells and (b) the presence of normal or immune serum did not significantly alter this result.

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