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Studies on the immune protection to murine experimental brucellosis conferred by Brucella fractions

I. POSITIVE ROLE OF IMMUNE SERUM

SIMONE BASCOUL, ARLETTE CANNAT, MARIE-FRANCE HUGUET & ARLETTE SERRE *Laboratoire d'Immunologie, Inserm V65 Institut de Biologie, Boulevard Henry IV,* 34000 *Montpellier*

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Summary. Mouse inoculation with three different phenol-insoluble fractions extracted from *Brucella melitensis* (fractions 'PI', '4A' and '5') induces an acceleration of the blood clearance of i.v. inoculated live Brucella and a diminution of the rate of multiplication of the injected bacteria in the spleen. Preincubation of the challenge inoculum in immune serum or i.p. injections of immune serum confer a good specific protection to non-immunized hosts. The results observed with fractionated sera suggest that, not only antibodies, but also other serum constituents may participate in the protective activity of immune sera. This is discussed in terms of the respective importance of humoral and cellular immunity to *Brucella* and of the choice of the best preparations for human or animal vaccination.

INTRODUCTION

'PI', a phenol extracted fraction from *Brucella melitensis* has been used for years in our laboratory for the vaccination of human workers at risk (Roux, Asselineau, Serre & Lacave, 1970). Further fractionation of PI leads to numerous immunogenic subfractions some of which also display protective properties against murine experimental Brucellosis (Lopez-Merino, Asselineau, Serre, Roux, Bascoul & Lacave, 1976; Lopez-Merino, 1976). However the mechanism of the protective activity of these and other 'vaccinating' fractions and the respective roles of humoral and cellular immunity have not yet been elucidated (Deppie, Witt & Smith, 1971; Lopez-Merino *et al.*, 1976; Rasooly, Olitzki & Sulitzeanu, 1966; Roux, Asselineau, Serre & Lacave, 1967). As a first step in this direction, we have in the present work studied the role of humoral immunity; after establishing criteria and conditions of immune protection by three different phenol insoluble fractions, we have shown that the serum from protected donors actually confers good protection on non-immunized hosts. Our results further suggest that, not only antibodies, but also other serum constituents, may participate in this protective activity.

Correspondence: Professor Arlette Serre, Laboratoire d'Immunologie, Institut de Biologie, Boulevard Henri IV, 34000 Montpellier, France.

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MATERIALS AND METHODS

Mice

Female outbred mice of the Swiss Webster line raised

in pathogen free conditions and aged 6–8 weeks were purchased from Iffa-Credo, France.

Brucella fractions
The extraction of fractions PI, 4A and 5 from *Brucella melitensis* M 15 has been previously described (Roux *et al.*, 1967; Lopez-Merino *et al.*, 1976; Lopez-Merino, 1976). Briefly, fraction PI is the phenol insoluble phase obtained after three phenol-water extractions at 65° (Westphal Method). Further purification of fraction 'PI' with DNase, RNase, pepsin, papain and pronase leads to fraction '4A', with DNase, RNase, papain and pronase (no pepsin) to fraction '5'. Fraction PI contains proteins, lipoproteins, sugars, amino-sugars and trace nucleic acids; so do fractions 4A and 5 but these have lost 90% of the proteins contained in PI. The peptidoglycan is linked to lipoproteins in all three fractions.

Immunization
This was obtained by one subcutaneous injection of a fraction homogenized in saline and without adjuvant. The doses used were 100 μg for PI, 10 or 100 μg for 4A and 5.

Blood samples
The samples obtained by retro-orbital puncture at different intervals were pooled and kept at $-20°$ until further use.

Anti-brucella antibodies
Their titre was estimated by indirect immunofluorescence as the last dilution giving clear-cut positive reactions using ethanol fixed smears of Brucella (*B. Swiss* strain 1330) and a commercial FITC conjugated anti-mouse immunoglobulin serum diluted 1/100 (Institut Pasteur, Paris, France).

Serum fractionation
Crude immunoglobulin fractions were prepared with 50% ammonium sulphate precipitation of pooled normal or immune sera.

They were further purified by three cycles of chromatography on ACA 34 ultragel (LKB Institute S.A. Orsay, France) in 0·01 M phosphate buffer 0·17 M NaCl pH 7·2 (Delay *et al.*, 1974).

The dialysed supernatant of ammonium sulphate precipitation of PI 30 serum was further purified by ultrafiltration on amicon membranes (Diaflo PM × 30 and XM × 100).

Heavy chain specific antimouse Ig sera (Miles Laboratories Ltd.) were used in double diffusion in Agarose 1·5% for the identification of Ig fractions and for the control of supernatants.

Challenge inoculation
Brucella Swiss 1330 cultivated on Albimi agar (Difco) were given i.v. at a dose of 2×10^5 live bacteria in 0·2 ml saline. 2×10^7 live *Listeria monocytogenes* were used as a challenge inoculum for specificity controls.

Preincubation of challenge bacteria with normal or immune serum
Equal volumes of a Brucella dilution and of normal or immune serum (or of the corresponding fractions) were incubated for 15 min at laboratory temperature and 0·2 ml of this mixture containing 2×10^5 viable bacteria and 0·1 ml of serum (or of the corresponding fractions) were used as challenge inoculum.

Passive transfer
0·2 ml of normal or immune serum (or of the corresponding fractions) were given i.v. 5, 3 and 1 days before challenge inoculation.

Blood clearance of Brucella
5, 10, 20 and 30 min after challenge inoculation samples of heparinized blood were obtained by retro-orbital puncture. Duplicate aliquots of 10 μl were cultured on Albimi agar plates and bacterial counts obtained on day 5.

Splenic infection rate
7 days after challenge inoculation (and in some experiments 2 or 20 days) the spleen were aseptically removed and homogenized. Duplicate aliquots of 10^{-5}, 10^{-3}, and 10^{-4} dilutions were cultured on Albimi agar and the bacterial counts estimated on day 5. In all experiments groups of four animals were used and each experiment was repeated at least twice.

RESULTS

Criteria and properties of protection following immunization with fractions PI, 4A and 5

Brucella infection is not lethal for mice. Numerous different criteria of immune protection have therefore been described for the study of experimental

Table 1. Blood clearance of Brucella in mice immunized with PI and 4A fractions

Time after immunization (days)	Time of sample collection (min)	Bacterial counts*			
		PI		4A	
		Controls	Immune mice	Controls	Immune mice
14	5	317	323	80	2
	10	235	15	72	1
	20	260	12	52	0
	30	350	2	60	0
30	5	459	14	71	20
	10	382	3	25	5
	20	309	9	2	1
	30	206	0	1	0
45	5	210	50	539	162
	10	200	15	414	21
	20	120	10	342	3
	30	107	4	200	1

* Mean of bacterial counts in duplicate aliquots of blood obtained from four mice (10 μl). For each time period after immunization the results of separate experiments include normal controls and immune mice.

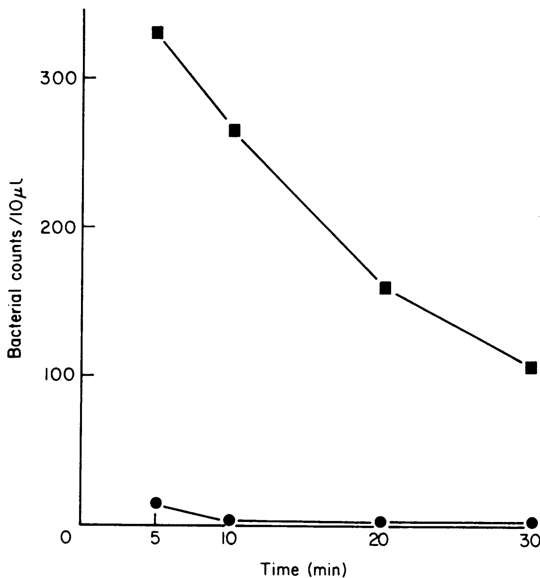

Figure 1. Blood clearance of i.v. inoculated Brucella in normal (■) and immune (●) mice.

brucellosis (Olitzki, 1970). In the present work we have chosen two criteria allowing a relatively rapid assessment of immune protection, together with an analysis of the early and the somewhat late events occurring during the immune response: the blood clearance of i.v. inoculated Brucella and the splenic infection index on day 7.

(a) *The blood clearance of i.v. inoculated Brucella* is accelerated in immunized mice. Figure 1 illustrates a typical clearance curve and shows that while in non-immunized controls the bacterial counts observed with blood aliquots obtained 5 to 30 min after i.v. challenge inoculation of 2×10^5 live Brucella remain high, a very rapid decrease in bacterial counts can be observed in immunized mice. Table 1 illustrates the kinetics of the effect of immunization on blood clearance; with fraction PI, the 'protecting effect' appears rather slowly and is maximal on day 30. With fraction 4A, although the 'protection' on day 30 still excellent, a maximal effect is obtained as soon as day 14. The effect of fraction 5 is very similar to that of PI and therefore not illustrated.

(b) *The splenic infection index.* Bacteria such as Listeria and Brucella are known to multiply in the spleen of infected animals. Thus in control mice the total number of splenic Brucella undergoes a rapid increase up to day 7 (followed by a very slow decrease). In immunized mice the total number of splenic bacteria on day 2 is lower than in controls and the factor of multiplication from day 2 to day 7 is lower so that a maximal difference in bacterial counts is observed on day 7. Figure 2 illustrates a

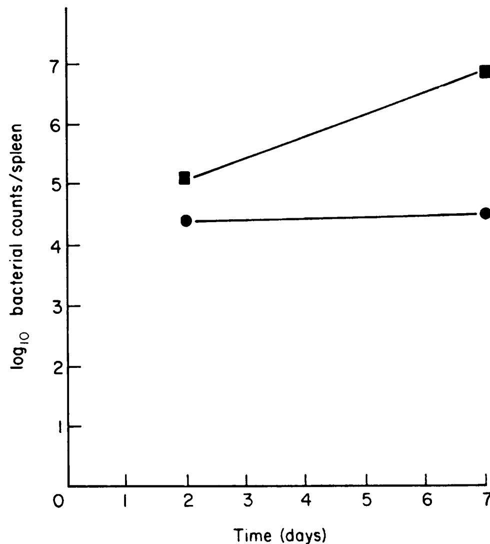

Figure 2. Multiplication of i.v. inoculated Brucella in the spleen of normal (■) and immune mice (●) between days +2 and +7 after challenge inoculation.

typical experiment; on day 2 the total number of splenic bacteria is of $2 \cdot 9 \times 10^4$ in immunized mice and of $12 \cdot 4 \times 10^4$ in controls; on day 7 the respective numbers are $3 \cdot 4 \times 10^4$ versus $6 \cdot 3 \times 10^6$ which corresponds to a multiplication factor of 51 in controls and of 1·2 in immunized mice. We have defined a splenic infection index (S.I.I.) as:

$$\frac{\text{Number of Brucella in immune spleen}}{\text{Number of Brucella in normal spleen}} \times 100$$

and measured protection by this S.I.I. on day 7 after challenge inoculation (S.I.I.7.). Table 2 shows that the 'best' S.I.I.7 are observed in PI 30 immunized mice.

Table 2. Splenic infection index on day 7

Time after immunization (days)	Immunization schedule			
	PI 100γ	4A 100γ	4A 10γ	5 100γ
14	4*	53	6	14
30	1	1	6	47
45	13	16	8	62
60	29	ND	45	ND

* Splenic infection index on day 7 = $\frac{\text{Number of Brucella in immune spleen}}{\text{Number of Brucella in normal spleen}} \times 100.$

(c) *The specificity of the protection* conferred by vaccination with these phenol insoluble Brucella fractions has been verified using *Listeria monocytogenes* as a challenge inoculum. With 2×10^7 live bacteria the blood clearance of injected Listeria and the 'listeria S.I.I.' are identical in normal and immunized mice.

Effect of immune serum on the response of non-immune mice to a challenge inoculation

This has been studied using two different methods, the preincubation of challenge bacteria with immune or non-immune serum, and the passive transfer of immune serum to non-immune mice.

(a) *Preincubation of challenge bacteria with immune or non-immune serum.* As seen in Table 3 the blood clearance of Brucella incubated with serum from PI 30 immunized donors (S.PI 30) is highly accelerated in the normal mouse, reaching a level similar to that observed in actively immunized mice. This striking similarity with the results obtained in actively immunized mice is also observed with S.PI 14 or S.PI 45 preincubated inocula. The S.I.I.7 obtained in the same conditions are shown in Table 4, together with a reminder of S.I.I.7 obtained in actively immunized mice (Table 2) and with the titres of anti-Brucella antibodies observed in the pooled sera used for preincubation. Several points should be underlined. The protective effect of an immune serum parallels the protection observed during active immunization: preincubation with S.PI 30 gives a lower S.I.I.7 than does preincubation

Table 3. Blood clearance of normal mice after inoculation with Brucella pre-incubated in PI 30 serum

Time after Brucella challenge (min)	Bacterial counts*	
	Brucella pre-incubated in normal serum (controls)	Brucella pre-incubated in PI 30 serum
5	870	70
10	400	2
20	500	0
30	600	0

* Mean of bacterial counts in duplicate aliquots of blood obtained from four mice (10 μl).

Table 4. S.I.I.7 in normal mice inoculated with Brucella pre-incubated in normal or immune serum as compared with S.I.I.7 after active immunization and with antibody titres in immune sera

Time after immunization (days)*	S.I.I.7 Normal mice Brucella pre-incubated in immune serum	S.I.I.7 Immune mice non pre-incubated Brucella	Antibody titre (IF)
14	14	4	10
30	4	1	80
45	8	13	20

* This relates either to the time when the immune serum used for pre-incubation was collected or to the time after active immunization.

Table 5. Total number of splenic Brucella in mice inoculated with Brucella preincubated in normal or immune serum

Time after immunization (days)	Brucella pre-incubated in immune serum	Brucella pre-incubated in normal serum
2	$4 \cdot 10^4$	$12 \cdot 10^4$
7	$1 \cdot 10^5$	$11 \cdot 10^6$
Splenic multiplication factor	2·5	91

with S.PI 14 or S.PI 45. A similar parallelism is observed between the protective effect of an immune serum and its antibody titres. Up to day 30 the S.I.I.7 obtained by preincubation are always somewhat higher than those observed in actively immunized mice. Moreover Table 5 shows that preincubation of the infective challenge organisms with immune serum not only leads to a lower (1/3) number of splenic Brucella on day 2 but also acts on the rate of multiplication of these splenic Brucella (2·5 versus 91).

(b) *Passive transfer of immune protection by serum injections.* Table 6 illustrates the results obtained in two representative experiments with normal mice receiving 0·2 ml of PI serum on days −5, −3, −1 prior to their challenge with live Brucella. It shows that blood clearance although highly accelerated as compared to the corresponding controls is not quite as rapid as that observed in actively immunized mice or with method 'a' especially as regards 20 and 30 min samples (see Tables 1 and 3). Nor are the S.I.I.7 of S.PI 30 transferred mice as low as those illustrated on Table 4: on the other hand S.PI 14 transferred mice have repeatedly shown very 'good' S.I.I.7. On the whole however there is no great difference between the results obtained by the two methods employed; this means that passive transfer of immune protection may be obtained with as little as 0·1 ml immune serum.

(c) *Controls.* The absence of a direct bactericidal effect of immune (or normal) serum has been verified.

Table 6. Blood clearance of Brucella and S.I.I.7 in normal mice after passive transfer of PI serum

Time after Brucella challenge (min)	Bacterial counts			
	Normal mice + Normal serum	Normal mice + PI 14 serum	Normal mice + Normal serum	Normal mice + PI 30 serum
5	393	15	659	49
10	391	6	580	12
20	350	6	370	7
30	247	6	400	6
S.I.I.7	3·5		17	

Table 7. Specificity controls: blood clearance of *Listeria monocytogenes* and S.I.I.2* in normal mice after passive transfer of normal or PI 30 serum or after inoculation with Listeria pre-incubated in PI 30 or normal serum

Time after Listeria challenge (min)	Bacterial counts			
	Listeria pre-incubated in normal serum	Listeria pre-incubated in PI 30 serum	Passive transfer of normal serum	Passive transfer of PI 30 serum
5	600	500	1000	1000
10	91	80	800	800
20	6	9	150	400
30	4	1	50	50
S.I.I.2	136		250	

* The splenic infection index was calculated on day +2 after challenge inoculation because of the rapid lethal effect of Listeria (with no difference between mice receiving normal or PI immune serum).

So has the specificity of the passive protection conferred by immune sera (see Table 7).

Studies with fractionated sera

Passive transfer of immune protection by the whole serum does not *per se* demonstrate the protective role of humoral antibodies. We have therefore prepared a crude Ig fraction from pooled PI 30 sera with ammonium sulphate precipitation. This was used for absorption tests and further purified on ACA 34 ultragel in 0·01 M phosphate buffer 0·17 NaCl pH 7·2 (Delay *et al.*, 1974). The crude Ig preparation and peaks 1(IgM+ IgG2−) and 2 (IgG2+ IgM−) were used at a concentration of 200 γ/ml for the preincubation of bacterial challenge inoculum.

(a) *Absorption test.* Absorption of the PI 30 crude Ig fraction with Brucella destroys its protective properties together with its weak antibody activity.

(b) *Preincubation of Brucella inoculum with fractionated Ig preparations.* Table 8 illustrates two such experiments. It shows that the crude Ig and the 7 S Ig fractions display protective properties evidenced

Table 8. Blood clearance of Brucella and S.I.I.7 in normal mice after inoculation with Brucella pre-incubated in fractionated PI 30 serum

Time after Brucella challenge (min)	Bacterial counts					
	Brucella pre-incubated in:					
	Normal serum (controls)	Crude Ig globulin fraction from PI 30 serum	Normal serum (controls)	PI 30 serum	Ig 19S fraction of PI 30 serum	Ig 7S fraction of PI 30 serum
5	490	404	328	84	331	273
10	490	60	264	9	116	86
20	240	12	162	1	37	17
30	100	3	104	0	10	5
S.I.I.7		21		4	63	18

Table 9. Blood clearance S.I.I.7 and Brucella multiplication rate observed in normal mice inoculated with Brucella pre-incubated in a < 100,000 Sn PI 30 fraction'

Time after Brucella challenge (min)	Bacterial counts	
	Brucella pre-incubated in normal serum	Brucella pre-incubated in ' < 100,000 Sn PI 30'
5	149	146
10	66	31
20	54	6
30	30	3
Splenic multiplication rate from day +2 to day +7	60	6
S.I.I.7	1·9	

both by acceleration of blood clearance and by S.I.I.7 values (the Ig 19S fraction is only very weakly protective). On the other hand the protection conferred by Ig fractions was repeatedly found to be lower than that conferred by the whole serum. The dialysed supernatant (Sn) of ammonium sulphate precipitation of PI 30 serum was therefore tested for protective properties. Preincubating the bacterial challenge with 'Sn PI 30' was shown to lead to fairly good protection in terms of S.I.I.7 (3·8, 10, 1·8 in successive experiments). Although 'Sn PI 30' does not precipitate in agar gel against specific IgG2 and IgG1 antisera, this does not eliminate the possible presence of trace Ig undetectable with this technique. 'Sn PI 30' was therefore filtered on amicon ultra filters (Diaflo PM × 30 and XM × 100). The protective activity was shown to correspond to fractions with a molecular weight between 30,000 and 100,000 (S.I.I.7 for fractions < 30,000 = 100 for fractions < 100,000 = 3). Table 9 shows that the < 100,000 Sn PI 30 fraction has but a weak effect on the blood clearance of preincubated Brucella. On the other hand it acts on the factor of multiplication of splenic Brucella (6 versus 60 between days +2 to +7 as compared to a multiplication factor of 3·3 versus 60 in the same experiment using the crude Ig fraction of the same PI 30 pool). Taken together these experiments show that not only antibodies but other serum constituents with molecular weights lower than those of intact immunoglobulins play a positive role in the immune protection to Brucella infection conferred by immunization with phenol insoluble Brucella fractions.

DISCUSSION

Our results show that the three phenol insoluble fractions used in this study (PI, 4A and 5) induce specific protection against murine experimental Brucellosis: their inoculation accelerates the blood clearance of i.v. injected Brucella and diminishes the rate of multiplication of the injected bacteria in the spleen. Differences in the kinetics of immune protection are observed between these fractions; they may be due to changes in immunogenic determinants, adjuvant like compounds or in the structure of the respective fractions. On the other hand, in a previous study (Roux *et al.*, 1967) using i.p. challenge inocula and comparing splenic infections on day +20, fraction 4A induced better S.I.I. than did fraction PI; the importance of the schedule used for comparing the properties of different fractions should be underlined.

It is clear from our results that the serum participates in this immune protection. Three questions however should be raised:

(a) Is this participation a constant feature, does it correspond to a phenomenon observable in man after either infection or vaccination?

(b) What is the mechanism of this serum protective activity?

(c) What is the respective importance of humoral and cellular immunity in this model?

Our experience in outbred and inbred mice is in favour of a constant positive participation of the serum in immune protection against murine experimental Brucellosis. Moreover several human sera from patients with clinical Brucellosis have been observed to transfer a good protection to normal mice (in terms of our criteria) and Sulitzeanu (1955) has described experiments of serum transfer of immune protection to Brucella infection in the guinea-pig. On the other hand a 'negative' 'facilitating like' effect has been shown by Forget, Borduas, Benoit, Turcotte, Meunier & Gusew-Chartrand (1977) with hyperimmunized chicken sera and by us with one human serum with IgA antibodies and a blocking activity on Wright's agglutination tests (unpublished results). The relative role of antibodies from different classes, affinities and functions and that of other serum constituents should therefore be investigated.

As regards the mechanism of serum protective activity the first hypothesis since crude Ig and purified IgG fractions are shown to act as 'protecting' factors is that of bacterial opsonization. The importance of opsonizing antibodies in other models of serum antibacterial protection is well documented: they are known to facilitate bacterial uptake by phagocytes (by way of Fc receptors for IgG Fc determinants on macrophages (Ehlenberger & Nussenzweig, 1976)) and thus bacterial elimination in liver and spleen. They could well be responsible for the lower number of live bacteria observed in this study during the early phases of Brucella infection. On the other hand, our results show that protective sera also act on the net rate of multiplication between days 2 to 7 (2·5 versus 91 with normal serum). This effect is probably not due solely to opsonization, antibodies with other functions and non-Ig factors or both may be involved. A similar phenomenon has been described by Ralston & Elberg (1969) who suggested that sera from rabbits immunized with whole Brucella activated normal macrophages and inhibited intra-cellular bacterial multiplication. Cahall & Youmans (1975) have ascribed a role in immune protection to *Mycobacterium tuberculosis* to non-immunoglobulin factors of immunity. The protective properties of serum fractions with molecular weight between 30,000 and 100,000, i.e. lower than that of intact immunoglobulins suggest that cellular immunity or co-operative factors could also play a role in the immune protection to Brucella: studies are underway to test this hypothesis.

The studies of Mackaness and his group have shown that, in the case of infections with facultative intracellular bacteria such as Listeria or Brucella, cellular immunity is central in the protection conferred against a secondary inoculation (Mackaness, 1964). This does not mean however that humoral immunity does not play an accessory role or that the mechanism of immune protection by vaccinating fractions and by viable bacteria is identical. As regards immunization with our fractions although serum constituents actually display protective properties the fact that the S.I.I.7 obtained in normal mice with immune sera or with Ig fractions are always higher than those observed in actively immunized mice point to a secondary role of humoral versus cellular immunity in the specific protection induced against Brucella infection. Cell transfer experiments in inbred mice and studies with 'Biozzi's high and low antibody producers' bring numerous arguments in confirmation of this hypothesis and emphasize the importance of macrophages (in preparation).

On the whole it would seem that humoral and cellular immunity both participate in the protection against experimental Brucellosis obtained with our fractions, and this should perhaps be taken into account in the choice of the best preparation for human or cattle vaccination.

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