In Vitro Studies on the Adjuvanticity of *Brucella* Fractions

ARLETTE SERRE,* JEAN-PIERRE VENDRELL, MARIE-FRANCE HUGUET, AND ARLETTE CANNAT

Laboratoire d'Immunologie, Institut National de la Santé et de la Recherche Médicale U 65, Institut de Biologie, 34000 Montpellier, France

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Two *Brucella* fractions, the murein-linked fraction PI and the murein-free fraction SF, behave as in vitro adjuvants for primary anti-sheep erythrocyte responses: added to Mishell and Dutton-type cultures of spleen cells from B6/DB F₁ mice they significantly enhance the number of direct anti-sheep erythrocyte PFC observed on day 5. They exert both nonspecific, polyclonal activating effects and antigen-dependent specific adjuvanticity. These two functions, however, differ in their dose responses and in their cellular requirements and can therefore be dissociated. Thus, polyclonal activation requires high doses of the "adjuvant fraction," is enhanced by adherent-cell depletion, and is not impaired by T-cell depletion. Specific adjuvanticity, on the other hand, requires lower doses of the adjuvant fractions (very high doses are in fact suppressive) and is T-cell and adherent-cell dependent. Moreover, adjuvanticity can be transferred to unstimulated spleen cells (or restored in adherent-cell-depleted populations) by PI- or SF-stimulated adherent cells or by the filtered supernatants of such cultures; adjuvant-soluble factors are therefore involved in the phenomena of adherent, T- and B-cell cooperation required for the adjuvanticity of *Brucella* fractions.

PI, a vaccinating fraction extracted from *Brucella melitensis* (24), displays strong adjuvant and immunostimulating properties (A. Escande, A. Serre, R. Senelar, and B. Serrou, Abstr. Soc. Fr. Immunol. Ann. Immunol. 129C:749, 1978). It compares favorably to *Mycobacterium bovis* BCG in these respects since in vivo inoculation of the *Brucella* fractions leads to a 4- to 10-fold increase in anti-sheep erythrocyte (SRBC) responses and to significant antitumoral effects. Although free from lipopolysaccharide (LPS), PI is a complex fraction and contains the *Brucella* peptidoglycan. Since the adjuvant effect of bacterial peptidoglycan is well documented (7), a comparison of the adjuvanticity of PI to that of another *Brucella* fraction, namely, the more purified PI-derived and murein-free fraction SF (A. Lopez-Merino, Ph.D. thesis, Université de Toulouse, Toulouse, France), seemed of some interest.

At the functional level both PI and SF behave not only as vaccines but also as B mitogens and polyclonal activators for murine lymphoid cells (29); whether these functions and adjuvanticity correspond to the same or to separable moieties is not clear, but a better understanding of such structural and functional relationships could lead to a better understanding of vaccines and adjuvants.

We have therefore undertaken in vitro studies on the adjuvanticity of these two *Brucella* frac-

tions in view of analyzing its scope, its cellular requirements, and its mechanisms. In the present paper we show that both fractions display in vitro adjuvanticity, that polyclonal activation (PCA) and specific antigen-dependent adjuvanticity can be dissociated in some, but not all, experimental conditions, and that specific adjuvanticity requires the participation of both T cells and adherent accessory cells.

MATERIALS AND METHODS

Mice. Female F₁ hybrids from C57BL/6 and DBA2 (B6/DB) and female nude mice on outbred Swiss background (together with their Nu/+ littermates) were purchased from IFFA CREDO France. They were raised in pathogen-free conditions and used at 6 to 8 weeks of age.

***Brucella* fractions.** The extraction of fractions PI and SF from *B. melitensis* biotype 1 has been described previously (Lopez-Merino, thesis; 24). Briefly, PI is the phenol-insoluble phase obtained after three phenol-water extractions at 65°C from delipidated bacteria. Further purification of fraction PI with DNase, RNase, pepsin, papain, pronase, and sodium dodecyl sulfate treatment gives rise to fraction SF. PI contains proteins, lipoproteins, sugars, amino sugars, and trace nucleic acids (24), whereas SF contains but two or three proteins and lipoproteins (Lopez-Merino, thesis). The *Brucella* peptidoglycan is covalently linked with lipoproteins in fraction PI and absent from fraction SF. Both fractions are insoluble in physiological conditions and therefore used as homogenized, tyndalized suspensions.

TABLE 1. Adjuvant effect of *Brucella* fractions on unseparated spleen cells^a

Spleen cells	PI (1 µg)	SF (0.1 µg)	SRBC	PFC/10 ⁶ viable cells	
				Expt no. 54	Expt no. 81
+	—	—	—	38 ± 9	67 ± 12
+	+	—	—	182 ± 27	84 ± 17
+	—	—	+	1,712 ± 188	2,241 ± 213
+	+	—	+	4,167 ± 516	3,333 ± 209
+	—	—	—	43 ± 1	
+	—	+	—	299 ± 9	
+	—	—	+	420 ± 105	
+	—	+	+	2,000 ± 102	

^a Data are given as the means of triplicate assays from duplicate cultures ± standard errors.

The *Brucella* LPS is restricted to the aqueous and phenolic phases of extraction by the method of Westphal. Contamination of fractions PI and SF by *Brucella* LPS or lipid A has nevertheless been explored and has been ruled out by the following: (i) the absence of sugars, glucosamine, and phosphorus in fraction SF and of β-hydroxymyristic acid in both fractions; (ii) the absence of PI-induced leucopenia shown by the method of Leong et al. (16); and (iii) the results of the toxicity test in adrenalectomized mice by the method of Galanos (10) and of the *Limulus* test (17) showing that endotoxin contamination is lower than 0.001 or 0.00005 µg, respectively, for 1 µg of PI or SF.

PI and SF behave as nonspecific B mitogens for mouse lymphocytes (29). Their polyclonal activating properties have been assessed by the following: (i) the fact that they enhance not only anti-SRBC background PFC but also total immunoglobulin secretions (measured by laser nephelometry by the method of Virella et al. [30]) and the percentage of immunoglobulin-secreting cells (estimated by immunofluorescence by the method of Gronowicz and Coutinho [11]); and (ii) the fact that in vivo injection of PI into C57BL/6 mice leads to an enhancement of seric autoantibodies such as anti-single-stranded DNA antibodies and rheumatoid factor (Cannat et al., submitted for publication).

Cultures. Mouse spleens were aseptically removed, and cell suspensions were prepared by passage through a sterile nylon filter. Cells were washed and cultured by a Mishell and Dutton method modified as described previously (22). Briefly, 2×10^7 spleen cells were cultured in 2 ml of medium (RPMI 1640; glutamine [GIBCO Laboratories] supplemented with 10% fetal calf serum and 5×10^{-5} M mercaptoethanol) in Nunclon petri dishes (35 mm) and incubated at 37°C in a humidified chamber containing 7% CO₂. Immunization was realized with 5×10^6 SRBC. The doses of *Brucella* fractions added to the culture and the time of this addition are detailed below. Cultures were assayed for direct anti-SRBC PFC on day 5 after determination of the number of viable spleen cells (trypan blue exclusion) by a modification (14) of Jerne's hemolytic plaque assay.

Cell separation procedures. Adherent populations and adherent-depleted populations were separated by incubation on Nunclon petri dishes (35 mm) at 37°C for 2 h. Nonadherent cells were removed by repeated addition and removal of medium (three to four times),

the remaining adherent monolayer serving as a source of adherent cells. In some experiments spleen cells were submitted to a 1,000-rad irradiation before cell separation.

T-cell-depleted spleen cells. T-cell-depleted spleen cells were obtained by treatment with an anti-θ serum (prepared in AKR mice by immunization with C3H/eb thymocytes) and with agarose-adsorbed rabbit serum as a complement source. Controls showed that about 30% of normal spleen cells and more than 99% of thymic B6/DB cells were killed under these conditions. The mixture was incubated either in glass tubes or directly in the Nunclon petri dishes used for the culture (in view of avoiding adherent-cell depletion).

Supernatants. Adherent cells were prepared from 10×10^6 spleen cells (normal or irradiated) and cultured for 18 h in 2 ml of medium containing 1 µg of PI or 0.1 µg of SF in a volume of 100 µl. Supernatants were pooled, centrifuged at 4,000 rpm for 20 min in the cold, dialyzed overnight against phosphate-buffered saline, filtered on 0.45-µm membrane filters (Millipore Corp.), and kept at -30°C until further use. Controls verified that passage through an anti-PI immunoabsorbent column by the method of Avrameas and Ternynck (2) did not impair their functional activity. Control supernatants were prepared in the same conditions, but without the *Brucella* fractions.

RESULTS

In vitro adjuvant activity of *Brucella* fractions. The addition of the phenol-insoluble *Brucella* fraction PI to Mishell and Dutton-type cultures enhanced the number of specific antigen-dependent direct anti-SRBC PFC (Table 1). Such was also the case with the more purified and peptidoglycan-free fraction SF (Table 1). This adjuvant effect was a constant phenomenon; thus, in 54 successive experiments 1.3 to 12.06 more PFC were observed in PI-stimulated cultures than in unstimulated cultures of spleen cells and SRBC ($P < 0.001$).

As regards kinetics, maximal adjuvant activity was observed in day 5 cultures where both the *Brucella* fractions and SRBC had been added to the spleen cells on day 0. However, a good adjuvant effect was also observed when the fractions and the SRBC were added to the cell culture on days 1 or 2 or when the cells were preincubated with the *Brucella* fractions for 24 h before the adjunction of SRBC on day 1.

In some, but not all, experiments, fractions PI and SF also enhanced PFC response obtained in the absence of added SRBC (Table 1). Although a total dissociation of this nonspecific activation (possibly due to the PCA properties of *Brucella* fractions; see above) and specific antigen-dependent adjuvant activity was not often obtained with cultures of total, unseparated spleen cells, the optimal doses of *Brucella* fractions for these two phenomena were different (Fig. 1). Concentrations of 10 µg of PI (or 1 µg of SF, data not shown) were optimal for nonspecific polyclonal activation of 10^7 spleen cells, and concentrations

FIG. 1. Effects of dose variation on the adjuvanticity of fraction PI. Symbols: ▨, without SRBC = polyclonal activation; □, with SRBC = antigen-specific response.

of 1 µg of PI (or 0.1 µg of SF) were optimal for specific adjuvanticity (and thus used in the present study). Moreover, supraoptimal doses of PI or SF (e.g., 100 µg of PI for 10^7 spleen cells) led to suppression of specific anti-SRBC response. This, together with the fact that fractions PI and SF are insoluble in physiological conditions (which leads to irregularities in the actual doses involved), could explain the variability of the adjuvant effects observed in otherwise comparable experiments.

T-cell requirement of the adjuvant effect of *Brucella* fractions. That T-cell depletion of a splenic population by treatment with anti-θ serum containing complement greatly impairs anti-SRBC responses is well documented (26). Such treatment also suppressed the specific antigen-dependent adjuvant effect of *Brucella* fractions (Table 2). Since the anti-θ serum and the complement solution alone had no influence on SRBC responses, the T-cell dependence of PI and SF specific adjuvant effect is strongly suggested. On the other hand, the nonspecific PCA effect of *Brucella* fractions was not impaired by T-cell depletion; it was even sometimes en-

hanced (Table 2). This, however, was not a constant phenomenon and may have been due to a partial adherent-cell depletion during incubations.

Spleen cells from homozygous nude mice, although they had very low anti-SRBC responses, were quite responsive to the adjuvant effects (both specific and nonspecific) of fraction PI (Table 3). Possible causes of the discrepancy observed between T-cell-depleted splenic populations from normal mice and spleen cells from nude mice are discussed below.

Adherent-cell dependence of PI and SF adjuvanticity. Table 4 shows the effects of adherent-cell depletion experiments. In adherent-cell-depleted populations, the PFC background and the polyclonal effect of *Brucella* fractions were always higher than those in unseparated populations. On the other hand, depletion of adherent cells inhibited antigen-dependent anti-SRBC response, even in the presence of fractions PI or SF. The specific adjuvanticity of *Brucella* fractions is therefore highly adherent-cell dependent.

Table 5 illustrates the results of preincubating adherent cells with *Brucella* fractions: adherent cells, preincubated for 18 h with PI (1 µg) and then thoroughly washed, transferred specific adjuvanticity to unstimulated adherent-cell-depleted spleen cells (and restored their anti-SRBC background response). As regards kinetics the minimum incubation time of adherent cells with PI was 6 h (data not shown). However, in standard conditions incubation was maintained for 18 h. X-irradiation (1,000 rads) of the adherent population before its incubation with the *Brucella* fractions did not impair the transfer of adjuvanticity.

On the other hand the addition of adherent cells preincubated with PI tended to inhibit the polyclonal effect of the fraction and thus to counterbalance the enhancing effect of adherent-cell depletion on this function.

Role of soluble factors. The filtered supernatant fluid of adherent cells cultured with the adjuvant fractions also enhanced the anti-SRBC response (both specific and nonspecific) of unstimulated cell populations (Table 6). Cell-to-cell

TABLE 2. Effect of T-cell depletion

Spleen cells	PI (1 µg)	SRBC	PFC/10 ⁶ viable cells			
			Untreated cells		Cells treated with anti-θ + complement	
			Expt no. 91	Expt no. 95	Expt no. 91	Expt no. 95
+	—	—	58 ± 13	22 ± 7	76 ± 3	34 ± 4
+	+	—	161 ± 24	121 ± 15	716 ± 107	132 ± 11
+	—	+	2,969 ± 252	308 ± 26	562 ± 67	92 ± 6
+	+	+	6,103 ± 427	4,294 ± 212	469 ± 41	158 ± 12

TABLE 3. Effect of fraction PI on anti-SRBC responses of nude mice

Spleen cells	PI (1 µg)	SRBC	PFC/10 ⁶ viable cells	
			Nu/+	Nu/Nu
+	—	—	132 ± 16	27 ± 5
+	+	—	153 ± 50	187 ± 8
+	—	+	479 ± 13	75 ± 14
+	+	+	1,471 ± 235	841 ± 176

cooperation among PI- (or SF)-stimulated adherent cells and total (or adherent-cell-depleted) unstimulated cells is therefore not necessary for the expression of *Brucella*-induced adjuvanticity, which involves the participation of soluble adjuvant factor(s).

DISCUSSION

Numerous bacterial products behave as in vivo adjuvants, but only several have been studied at the in vitro level. Such is the case for nonpurified crude extracts from *Listeria monocytogenes* (4) or for purified fractions such as NWSM from *Nocardia* spp. (6) or trehalose dimycolate from *Mycobacterium* spp. (15). Since the bacterial peptidoglycans and their natural or synthetic muramyl dipeptides (7, 8, 14, 27) and the LPSs of gram-negative bacteria (5, 26) are the best documented in this respect, the possible role of these two ubiquitous bacterial components in the phenomena observed with *Brucella* fractions was open to discussion. Of the two *Brucella* fractions used in this study, one, PI, contains the *Brucella* peptidoglycan; the fact that the murein-free fraction, SF, displays comparable adjuvant effects suggests, however, that the murein moiety is not responsible for its adjuvanticity. Similarly, the absence of LPS contamination (see above) in both PI and SF suggests that their adjuvanticity could in fact be due to the lipoproteins which represent the essential constituents of fraction SF and the major constituents of fraction PI. These lipoproteins are biochemically comparable to the lipoproteins of the outer membrane of enterobacteria described by Braun and Rehn (3). The B mitogenicity and the polyclonal activating effect of these lipoproteins have been described previously (20), but not, to our knowledge, their

TABLE 5. Transfer of specific adjuvanticity to an adherent-cell-depleted population by adherent cells preincubated with PI

Day 1		Day 0		Day 5
Adherent spleen cells	PI (1 µg)	Adherent-cell-depleted cells	SRBC	PFC/10 ⁶ viable cells
+	—	+	—	177 ± 14
+	+	+	—	216 ± 8
+	—	+	+	460 ± 13
+	+	+	+	2,582 ± 280

adjuvanticity on specific antigen-dependent responses.

Correlation between polyclonal and nonspecific adjuvanticity has often been reported, and the problem of the unity or independence (or both) of these two functions has often been evoked (22, 23). Unity is now disproved by numerous experiments showing that dissociation of PCA and specific adjuvanticity is obtainable by dose variation (7, 27); such is also the case in the present study where the doses of *Brucella* fractions optimal for PCA are about 10 times higher than those optimal for specific adjuvanticity. We show moreover that the T-cell and adherent-cell requirements of these two phenomena are quite different; PCA does not require mature T cells and is not impaired (but, on the contrary, is enhanced) by adherent-cell depletion, whereas specific adjuvanticity requires the presence of both T and adherent cells. This does not mean that in physiological in vivo conditions or in in vitro experiments with total unseparated cell populations PCA does not play a role in specific adjuvanticity.

T-cell dependence of in vitro adjuvanticity has been evidenced for LPS (1, 19) and for muramyl dipeptide (18). On the other hand the adjuvanticity of the crude *Listeria* fraction, LCWF, is described as T-cell independent (4) and therefore possibly due to different mechanisms. A discrepancy has been observed in the present study and in that of Watson and Whitlock with muramyl dipeptide (31) between the apparent T-cell dependence of in vitro adjuvanticity when estimated by T-cell depletion of the normal splenic population by anti-θ serum containing complement and its apparent T-cell indepen-

TABLE 4. Effects of adherent-cell depletion

Total spleen cells	PI (1 µg)	SRBC	PFC/10 ⁶ viable cells	Adherent-depleted spleen cells	PI (1 µg)	SRBC	PFC/10 ⁶ viable cells
+	—	—	10 ± 3	+	—	—	112 ± 14
+	+	—	120 ± 20	+	+	—	2,220 ± 205
+	—	+	3,916 ± 287	+	—	+	28 ± 5
+	+	+	8,941 ± 421	+	+	+	71 ± 2

TABLE 6. Adjuvant effect of supernatants from PI- (or SF)-stimulated adherent cells

Spleen cells	SN/PI ^a (100 μ l)	SN/SF ^b (100 μ l)	SN/T ^c (100 μ l)	SRBC	PFC/10 ⁶ viable cells	
					Expt no. 55	Expt no. 85
+	—	—	—	—	112 $\pm$ 24	44 $\pm$ 6
+	—	—	—	+	412 $\pm$ 10	3,280 $\pm$ 180
+	+	—	—	—	298 $\pm$ 39	947 $\pm$ 18
+	+	—	—	+	1,963 $\pm$ 310	6,640 $\pm$ 451
+	—	+	—	—	245 $\pm$ 21	201 $\pm$ 14
+	—	+	—	+	1,798 $\pm$ 58	5,615 $\pm$ 343
+	—	—	+	—	96 $\pm$ 13	ND ^d
+	—	—	+	+	549 $\pm$ 39	ND

^a SN/PI, Supernatant of adherent spleen cells preincubated with PI (see text).

^b SN/SF, Supernatant of adherent spleen cells preincubated with SF.

^c SN/T, Supernatant of adherent spleen cells cultured without adjuvant.

^d ND, Not determined.

dence when estimated by the adjuvant effect observed with spleen cells from nude mice. This could mean that T-cell or pre-T-cell populations present in the nude homozygous mice could be the effectors of adjuvanticity in these two models, a phenomenon already shown for other functions such as cytotoxicity (12).

In the present work with B6 $\times$ DB F₁ mice adherent cells were shown to be central for the adjuvant effects of *Brucella* fractions; adherent-cell depletion suppressed specific adjuvanticity, whereas on the other hand, specific adjuvanticity could be transferred to normal or adherent-depleted unstimulated cell populations by PI- or SF-stimulated purified and irradiated adherent cells. This phenomenon of adherent-cell dependence has also been observed with muramyl dipeptides (9) and LPS (27, 28). In most cases the adherent cells involved were peritoneal exudate cells and described as macrophages. In the present study, the cells involved were splenic adherent cells; their radioresistance suggests that they could be macrophages, but they are never in our hands 100% phagocytic or 100% esterase or peroxidase positive, so that the possibility of coexistence of macrophages and a small number of radioresistant T cells is not excluded.

The action of these adherent cells is clearly complex. Supraoptimal doses of the adjuvant fractions lead to diminution and even suppression of their adjuvanticity; on the other hand, adherent-cell depletion leads to a considerable enhancement of the nonspecific polyclonal activation together with a total suppression of antigen specific adjuvanticity. PI and SF therefore seem to stimulate both amplifying and suppressing of adherent cells so that the data observed with a given dose and with total splenic populations are but the results of specific and nonspecific effects on these different subpopulations. Studies are currently underway to analyze the stimulation of these different adherent cells by

PI and SF, the nature of their receptors, and the implication of phagocytosis and activation.

On the other hand, the present study has already shown that physical interactions between PI- or SF-stimulated adherent cells and the effector B (or helper T) cells are not indispensable for the adjuvant effect of these fractions; the supernatant fluid of adherent cells cultured with PI (or SF) mimics the action of such cells for both polyclonal and specific adjuvanticity. The complexity of such adjuvant supernatants and the relationships of soluble adjuvant factors with IL1 and other monokines will be the subject of a subsequent paper.

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