INFECTION AND IMMUNITY, July 1970, p. 1-6

Vol. 2, No. 1
Printed in U.S.A.

Influence of *Brucella* Endotoxins on the Initiation of Antibody-Forming Spleen Cells in Mice Immunized with Sheep Red Blood Cells

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Received for publication 9 February 1970

Changes in the formation of antibodies to sheep red blood cells (sRBC) in the presence of *Brucella* extracts was studied in mice whose spleen cells were assayed by the Jerne procedure. Two strains of female mice were employed. *Brucella* extracts were prepared: (i) by trichloroacetic acid extraction (LPSN), (ii) by phenol extraction (LPS), and (iii) by hot acetic acid hydrolysis (Ps). *B. abortus* LPSN and *B. melitensis* LPSN or LPS, administered with sRBC, stimulated the specific response to sRBC, but only at high doses of endotoxins. *B. abortus* LPSN and *B. melitensis* LPSN suppressed nonspecific responses against horse red blood cells (hRBC), in contrast to the typical events following administration of *Serratia marcescens* endotoxin (or endotoxins from other ubiquitous organisms). In CD-1 mice, *B. abortus* Ps depressed the specific anti-sRBC response. Attempts to presensitize mice with *abortus* LPSN resulted in a stimulation of the response to sRBC, but pretreatment with *B. melitensis* LPSN had an inhibitory effect. When injected alone, *Brucella* endotoxins activated anti-sRBC antibody-forming cells but not anti-hRBC cells. *B. abortus* Ps was unable to modify the background number of anti-sRBC cells and inhibited the hRBC response. These data suggest (i) that there exists a "common antigen" between *Brucella* cells and sRBC and (ii) that the so-called primary response to endotoxins from ubiquitous organisms represents a secondary response to already naturally sensitized animals.

Endotoxins from ubiquitous gram-negative organisms, when injected into an animal together with another antigen, e.g., sheep red blood cells (sRBC), enhance specific responses and at the same time produce a nonspecific activation of many different types of antibody-forming cells (6, 10–13, 17). In addition to their many other properties, such endotoxins can alter cell permeability (4, 18, 28) and may trigger the release of oligonucleotides that are capable of stimulating cells involved in antibody formation (7). In general, ubiquitous gram-negative organisms produce endotoxins that are toxic, pyrogenic, and sensitizing (17).

Brucella endotoxins, that is, extracts from nonubiquitous organisms, do not behave in the same manner. Normal animals respond poorly to endotoxins of *Brucella* (1, 11, 16) and cannot be sensitized so that typical endotoxin responses follow the injection of killed cells or *Brucella* endotoxins (16). Similarly, killed cells in incomplete Freund's adjuvant do not induce skin sensitivity in guinea pigs (24). When *Brucella* endotoxin in complete Freund's adjuvant does lead to typical endotoxin sensitivity in mice or in rabbits (11), the response is probably due to the well-known ability of mycobacterial cells or extracts to induce allergy not only against themselves but also against every antigen to which mycobacteria, or extracts thereof, are added (30). Ordinarily, prior exposure of the animal to live *Brucella* cells is a prerequisite for reactivity to *Brucella* or *Brucella* endotoxins (1, 2, 4, 5, 16).

The question remains whether such findings are due to true differences in the pharmacological properties of *Brucella* endotoxins and enterobacterial endotoxins or to differences in the probability of the animal's prior exposure to the antigen involved.

Responses measured in terms of the number of antibody-forming cells in the spleen of mice that were injected with sRBC as antigen and simultaneously received *Brucella* extracts, either for the first time or following attempted sensitization to these extracts, may help to answer this question.

MATERIALS AND METHODS

All tests involved the initiation of antibody response in vivo and its modification in the presence of endotoxins. We studied the response of mice spleen cells

by the procedure of Jerne (15) as modified by Nakano and Braun (19). We prepared suspensions of single spleen cells by teasing the tissue in Eagle's minimal essential medium (MEM) and subsequently passing the separated cells through stainless-steel mesh. A 0.2-ml portion of the spleen cell suspension was then mixed with 2 ml of 0.8% Noble Agar melted in MEM (plus 2 mmoles of L-glutamine per ml) at 46 C, and 3×10^8 freshly washed erythrocytes were added. The mixture was immediately poured into a 100-mm plastic petri dish and allowed to solidify. After 1 hr of incubation at 37 C, each plate was flooded with 1.5 ml of guinea pig complement diluted 1:5 in normal complement buffer, and the dishes were again incubated for 1 hr at 37 C. Hemolytic plaques were counted, and their frequency per 10^8 nucleated spleen cells was calculated.

We performed all assays 48 hr after the inoculation of mice. Plates containing sRBC and also plates containing horse RBC (hRBC) were used in the assays. Such double checks were performed to determine the specificity or nonspecificity of the response.

sRBC and hRBC were harvested in modified Alsever's solution. Prior to use, they were washed three times in sterile saline and then adjusted to the desired concentration by photometric measurement at 541 nm.

We employed two strains of mice: female CF-1 mice weighing about 20 g and female CD-1 mice (Caesarean-delivered, Charles River) of similar weight. Groups of at least five animals were used in all tests.

All injections were made intravenously in volumes of 0.2 ml. As antigen, we used a single dose of 10^8 sRBC.

We prepared three *Brucella* extracts as follows. (i) Endotoxins were extracted from smooth *B. abortus* strain 99, or from smooth *B. melitensis* strain 53 H 38, by the Boivin trichloroacetic acid technique (3); such endotoxins will be referred to, respectively, as LPSNA (*B. abortus*) and LPSNM (*B. melitensis*). (ii) An endotoxin was extracted from smooth *B. melitensis* strain 53 H 38 by the technique of Westphal and Lüderitz as modified by O'Neill and Todd (22); this preparation will be referred to as LPSM. (iii) A fraction which was obtained from smooth *B. abortus* strain 99 by hot acetic acid hydrolysis (14) and precipitated with 7 volumes of ethyl alcohol will be referred to as PsA. Sugar analysis of these extracts revealed that (25): *B. melitensis* lipopolysaccharide (LPSM) = glucose; *B. abortus* lipopolysaccharide (LPSNA) = glucose, mannose, and glucosamine; and *B. abortus* polysaccharide (PsA) = glucose (mannose) and glucosamine. We employed *Serratia marcescens* endotoxin (Difco) as a control for checking the well-known specific and nonspecific effects of endotoxins from ubiquitous gram-negative bacteria on antibody formation.

RESULTS

Effects of B. abortus extracts in CF-1 mice. Intravenous injection of a mixture of sRBC and 10 μg of LPSNA did not produce any significant modification of the response obtained after immunization of CF-1 mice with sRBC alone (Table 1). If anything, there was an inhibitory effect, but it was at the limit of significance. However, the nonspecific hRBC plaque-forming cells were inhibited. When 10 μg of PsA was mixed with sRBC, the specific response to sRBC was not modified; the nonspecific hRBC response also remained unchanged. When injected alone into CF-1 mice, 10 μg of LPSNA appeared unable to modify the background numbers of plaque-forming spleen cells for sRBC or hRBC which existed in untreated control mice.

TABLE 1. *Influence of Brucella extracts on the activation of specific sRBC antibody-forming cells and on the nonspecific response to hRBC when tested in CF-1 mice*

Treatment[a]	Plaque-forming cells/10^8 spleen cells (± SE) assayed on	
	sRBC	hRBC
None	25 ± 10.0	4 ± 1.6
sRBC	290 ± 55.6	9 ± 3.4
LPSNA (10 μg) + sRBC	225 ± 25.0	0
LPSNA (10 μg)	13 ± 4.8	9 ± 6.4
PsA (10 μg) + sRBC	337 ± 62.8	12 ± 5.9
LPSM (10 μg) + sRBC	125 ± 27.6	20 ± 8.8
LPSM (10 μg)	29 ± 20.0	0
LPSmar (10 μg) + sRBC	3,384 ± 121.6	129 ± 24.8
LPSmar (10 μg)	117 ± 50.8	82 ± 66.2

[a] LPSNA = trichloroacetic acid extract from *B. abortus*; LPSM = phenol extract from *B. melitensis*; PsA = hot acetic acid extract from *B. abortus*; LPSmar = endotoxin (Difco) from *S. marcescens*; sRBC = 10^8 sRBC in a volume of 0.2 ml.

Effects of B. melitensis extracts in CF-1 mice. When injected together with sRBC, 10 μg of LPSM decreased the number of specific sRBC plaque-forming cells by one-half but did not alter the number of nonspecific hRBC hemolytic plaques (Table 1). When injected alone into CF-1 female mice, 10 μg of LPSM did not interfere with the background number of sRBC plaque-forming spleen cells but inhibited the hRBC background population. In contrast to this result, the background number of hRBC plaque-forming spleen cells was observed to be unaltered after the injection of *B. abortus* LPSNA.

Effects of S. marcescens endotoxin in CF-1 mice. The well-known influence of endotoxins from ubiquitous organisms on the initiation of specific responses as well as nonspecific responses, was confirmed (Table 1). When injected alone or together with sRBC, 10 μg of *S. marcescens* LPS strongly stimulated both the specific sRBC and the nonspecific hRBC responses.

Influences of sensitizing pretreatments in CF-1 mice. We pretreated mice by injecting *B. abortus*

TABLE 2. *Influence of pretreatment by Brucella endotoxins[a] on the activation of specific sRBC antibody-forming cells and on nonspecific hRBC responses in CF-1 mice*

Pretreatment[b]	Treatment[b]	Plaque-forming cells/10^8 spleen cells (± SE) assayed on	
		sRBC	hRBC
None	None	39 ± 5.1	18 ± 5.6
None	sRBC	198 ± 42.0	18 ± 4.4
LPSNA (10 μg)	LPSNA (10 μg) + sRBC	308 ± 61.4	19 ± 5.4
LPSNA (10 μg)	LPSNA (10 μg)	39 ± 7.7	ND[c]
PsA (10 μg)	PsA (10 μg) + sRBC	297 ± 60.2	8 ± 3.3
PsA (10 μg)	PsA (10 μg)	28 ± 8.6	18 ± 5.3
LPSM (10 μg)	LPSM (10 μg) + sRBC	287 ± 89.0	19 ± 3.4
LPSM (10 μg)	LPSM (10 μg)	29 ± 5.8	11 ± 2.9

[a] Pretreatment 15 days before subsequent treatments.
[b] LPSNA = trichloroacetic acid extract from *B. abortus*; PsA = acetic acid extract from *B. abortus*; LPSM = phenol extract from *B. melitensis*; sRBC = 10^8 sRBC in a volume of 0.2 ml.
[c] Not done.

or *B. melitensis* endotoxins intravenously 15 days prior to the second injection of endotoxin. After pretreatment with 10 μg of LPSNA, treatment of CF-1 female mice with 10 μg of LPSNA alone still did not modify the background number of sRBC antibody-forming cells (Table 2). Similar pretreatment followed by injection of sRBC and 10 μg of LPSNA resulted in a slight, but probably insignificant, increase of the specific response to sRBC observed when sRBC and endotoxin were injected for the first time. This pretreatment did not influence the "normal" hRBC responses (Tables 1 and 2). Pretreatment with 10 μg of PsA failed to induce any changes either in the background numbers, compared with the untreated control mice, or in the number of specific and nonspecific plaque-forming cells produced after sRBC injections. Neither the specific sRBC response nor the nonspecific hRBC response was altered in CF-1 female mice pretreated with 10 μg of *B. melitensis* endotoxin (Table 2).

Effects of B. abortus extracts in CD-1 mice. Simultaneous intravenous injections of sRBC and 10 or 50 μg of LPSNA resulted in a slight depression of the number of anti-sRBC plaque-forming spleen cells in comparison with the effect of injecting sRBC alone (Table 3). However, when 100 μg of LPSNA was injected together with sRBC, a stimulation of the response to sRBC occurred. All three doses (10, 50, and 100 μg) of LPSNA injected with sRBC completely inhibited the formation of antibodies to hRBC by the background spleen cells, which occurs in normal mice and which was slightly stimulated after immunization with sRBC alone. Responses of antibody-forming spleen cells after intravenous injections of 10 to 100 μg of PsA plus sRBC resulted in a marked inhibition of the number of plaques specific for antibodies against sRBC. In the same groups of mice, the appearance of anti-hRBC plaques was completely suppressed. These results differed strikingly from that observed in similarly treated CF-1 female mice (Table 1). When injected alone in CD-1 mice, 100 μg of LPSNA activated cells making antibodies against sRBC but did not produce any detectable response in assays with

TABLE 3. *Influence of Brucella extracts on the activation of specific sRBC antibody-forming cells and on nonspecific responses to hRBC when tested in CD-1 mice*

Treatment[a]	Plaque-forming cells/10^8 spleen cells (± SE) assayed on	
	sRBC	hRBC
None	45 ± 7.9	5 ± 2.2
sRBC	531 ± 53.6	19 ± 7.8
LPSNA (10 μg) + sRBC	460 ± 85.7	0
LPSNA (50 μg) + sRBC	430 ± 17.5	0
LPSNA (100 μg) + sRBC	2,902 ± 247.9	0
LPSNA (100 μg)	113 ± 19.7	0
PsA (10 μg) + sRBC	87 ± 39.0	0
PsA (50 μg) + sRBC	99 ± 31.0	0
PsA (100 μg) + sRBC	123 ± 15.4	0
PsA (100 μg)	24 ± 5.4	0
None	20 ± 3.4	5 ± 1.2
sRBC	513 ± 28.2	11 ± 3.5
LPSNM (10 μg) + sRBC	261 ± 98.6	0
LPSNM (50 μg) + sRBC	701 ± 283.4	0
LPSNM (100 μg) + sRBC	1,063 ± 214.0	0
LPSNM (10 μg)	12 ± 7.7	0
LPSNM (50 μg)	102 ± 31.4	0
LPSNM (100 μg)	154 ± 2.6	0
LPSNM (200 μg)	25 ± 18.4	0

[a] LPSNA = trichloroacetic acid extract from *B. abortus*; PsA = acetic acid extract from *B. abortus*; LPSNM = trichloroacetic acid extract from *B. melitensis*; sRBC = 10^8 sRBC in a volume of 0.2 ml.

TABLE 4. *Influence of pretreatment by Brucella endotoxins[a] on the initiation of specific sRBC antibody-forming cells and on nonspecific hRBC responses in CD-1 mice*

Pretreatment[b]	Treatment[b]	Plaque-forming cells /10^8 spleen cells (± SE) assayed on	
		sRBC	hRBC
None	None	22 ± 3.4	5 ± 1.2
None	sRBC	513 ± 29.2	11 ± 3.5
None	LPSNA (50 μg) + sRBC	460 ± 85.7	0
LPSNA (50 μg)	LPSNA (50 μg) + sRBC	903 ± 62.5	12 ± 1.6
None	LPSNM (50 μg) + sRBC	701 ± 283.4	0
LPSNM (50 μg)	LPSNM (50 μg) + sRBC	453 ± 14.1	0

[a] Pretreatment 15 days before subsequent treatments.
[b] LPSNA = trichloroacetic acid extract of *B. abortus*; LPSNM = trichloroacetic acid extract of *B. melitensis*; sRBC = 10^8 sRBC in a volume of 0.2 ml.

hRBC. However, 100 μg of PsA injected alone was unable to modify the background number of sheep erythrocyte plaque-forming spleen cells but still inhibited the background number of hRBC plaque-forming cells.

Effects of B. melitensis extracts in CD-1 mice. Intravenous injections of 10 μg of LPSNM together with sRBC, in CD-1 mice, led to a decrease in the specific response against sRBC and, in addition, completely abolished the nonspecific response to hRBC (Table 3). When the dose of LPSNM injected into CD-1 mice was increased, then a stimulatory effect of LPSNM appeared after simultaneous administration of sRBC. Only the anti-sRBC antibody-forming cell population was affected in this manner; anti-hRBC plaque-forming cells were inhibited. In this regard, *B. abortus* and *B. melitensis* endotoxins behaved similarly. When injected alone into CD-1 mice, 10 μg of LPSNM did not affect the background number of sRBC units, but it inhibited the hRBC response. While still inhibitory for the background cells forming antibody to hRBC, 50 or 100 μg of LPSNM, injected alone, activated antibody-forming spleen cell populations making antibodies to sRBC. The injection of 200 μg of LPSNM alone, however, was unable to stimulate any sRBC response, a rather unexpected finding.

Effects of sensitizing pretreatments in CD-1 mice. We pretreated CD-1 mice by injecting *B. abortus* or *B. melitensis* endotoxins intravenously 15 days prior to the second injection of endotoxin. Pretreatment of CD-1 female mice with 50 μg of LPSNA (*B. abortus*) stimulated the specific response to sRBC (Table 4). This mode of sensitization also restored the nonspecific hRBC response to a level comparable to that observed in control nonsensitized but sRBC-injected mice. As noted earlier, this response was abolished in mice that had been subjected to a single injection of the same *B. abortus* endotoxin (Table 3). In contrast, pretreatment with 50 μg of *B. melitensis* LPSN did not induce any specific stimulation and did not overcome the inhibition of the hRBC antibody-forming spleen cell populations.

DISCUSSION

The foregoing data indicate that only rather large amounts of *Brucella* endotoxins can increase the specific response, i.e., the number of anti-sRBC antibody-forming cells in the spleen of mice injected with sRBC and *Brucella* endotoxins. Whereas 10 μg of *S. marcescens* endotoxin sufficed to enhance specific or nonspecific antibody response, 100 μg of *Brucella* endotoxin was needed to obtain merely a stimulation of the specific anti-sRBC response.

Contrary to the typical events following administration of endotoxins from ubiquitous gram-negative bacteria (8–10), *Brucella* endotoxins, in vivo as in vitro (Sendt et al., *unpublished data*), failed to stimulate antibody-forming cell populations nonspecifically, i.e., the anti-hRBC antibody-forming cells, whose number is greatly increased in the spleen of mice injected with sRBC and *S. marcescens* endotoxin. Furthermore, *Brucella* endotoxins completely abolished the nonspecific response to hRBC, that is, the background number of such antibody-forming cells present in normal animals. In our assays, differences were noted between the responses of CF-1 and CD-1 mice. The latter strain always gave evidence of a complete inhibition of nonspecific hRBC responses, but this effect was not so clear-cut in CF-1 mice. These differences may be due to genotypic characteristics of the animals or to a dissimilar bacterial flora in the two strains of mice.

Pretreatment with *B. abortus* LPSN resulted in a degree of specific stimulation of antibody-forming cells and restored the nonspecific hRBC background number which was suppressed after

simultaneous injection of sRBC and LPSNA in normal untreated mice. This may be interpreted as a sensitization. The importance of the protein content of endotoxins in determining host reactivity has been suggested by others (12), and sensitization by *B. abortus* LPSN may be due to the proteinaceous component of this extract. However, no such effect was observed when mice were pretreated with *B. melitensis* LPSN (Table 4). On the contrary, sensitization by *B. melitensis* LPSN lowered the number of plaque-forming spleen cells compared with that observed in mice which were not pretreated. It may well be that a better knowledge of the chemical composition and of the stereoconfiguration of bacterial extracts will eventually help in explaining the mode of action of different antigenic fractions in specific and nonspecific antibody initiation.

When injected alone, either *B. abortus* or *B. melitensis* endotoxins activated spleen cells that produce antibodies against sRBC. However, the apparent incapacity of 200 μg of *B. melitensis* LPSN to produce the same kind of response remains without explanation. *B. abortu* P. s alone appeared to be ineffective and, when injected simultaneously with sRBC, inhibited the expected specific response.

These latter findings may be explained by postulating the existence of a common antigen (CA) between *Brucella* and RBC, a phenomenon similar to that uncovered by Neter (21) among different *Enterobacteriaceae*. CA from *Enterobacteriaceae* is a polysaccharide whose immunogenicity is suppressed when CA is complexed with other bacterial fractions prior to immunization (21). CA from gram-positive organisms has also been described with prroperties similar to those of CA from gram-negative bacteria (29). Thus, CA are different from the cross-reactive antigens for RBC and gram-negative or gram-positive bacteria which have already been described (23, 26, 27).

The existence of a factor analogous in properties to CA, common to *Brucella* cells and RBC, may explain the activation of the sRBC antibody-forming spleen cell population which follows the injection of *Brucella* endotoxins alone and may explain, too, the depression of such anti-sRBC cells after intravenous injection into mice of mixtures of *B. abortus* Ps and sRBC.

All of the above data may help in understanding the role of bacterial endotoxins in stimulating specific and nonspecific immunities (8, 9).

The so-called primary response, following injections of endotoxins from ubiquitous organisms, actually is a false primary response. In fact, it is always a secondary response, because normal adult animals, including man, have been subjected, throughout their life, to a variety of such antigenic stimuli. These ubiquitous endotoxins possess "dispersing properties" (i.e., the ability to convert a specific response into a nonspecific one) that may be attributed to antigen-antibody reactions involving lymphocytes of the already sensitized animals (8). At the same time, such reactions may release stimulatory oligonucleotides (9, 20). Dispersing properties and release of stimulatory oligonucleotides lead to nonspecific responses and help to increase the specific stimulation following injection of an antigen. Furthermore, preexistence of appropriate memory cells, elicited by prior contact with cross-reacting antigens, also may be a cause of the observed stimulations by ubiquitous endotoxins (e.g., enterobacterial lipopolysaccharide), and may involve a preexistence of sensitized lymphocytes (9).

Brucella endotoxins cannot trigger similar effects because "normal" mice do not "know" *Brucella* antigens. This immunological ignorance is demonstrated by the absence of positive responses in normal adult animals subjected to *Brucella* endotoxins (1, 11, 16) or to killed *Brucella* cells (16, 24) and by the typical endotoxin response observed when the animals were first *Brucella*-infected (1, 2, 4, 5, 16).

Sensitization is the cause of the so-called "typical" events following administration of endotoxins from ubiquitous gram-negative bacteria. The absence of a similar response in *Brucella* lipopolysaccharide-injected normal mice indicates that this is a true primary response: when a specific antigen, e.g., *Brucella* endotoxin, is an "unknown" one and is introduced for the very first time into an animal, the response is specific and then is limited to activation of specific antibody-forming clones. *Brucella* endotoxins do not possess any dispersing effect because no prior cytophilic *Brucella* antibodies exist in normal mice.

ACKNOWLEDGMENTS

We thank W. Braun, Institute of Microbiology, Rutgers, the State University, New Brunswick, N.J., for friendly and helpful advice, criticism, and editorial changes.

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