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Stimulating activity of *Brucella* fractions in a human lymphocyte transformation test

CORRELATION WITH HUMORAL AND CELLULAR IMMUNITY

SIMONE BASCOUL, M. PERALDI, AHIDÉ LOPEZ MERINO, CHARLOTTE LACAVE, ARLETTE CANNAT & ARLETTE SERRE *INSERM U.65, Montpellier, France*

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Summary. 'PI' a phenol-extracted fraction from *Brucella melitensis* has been used for years in our laboratory for the vaccination of laboratory and agricultural workers considered 'at risk'.

In the present work it has been used *in vitro*, together with another phenol-extracted and peptidoglycan containing fraction (4A) in a lymphocyte-tranformation test.

Correlation between stimulation index, humoral antibodies to *Brucella* and the cutaneous response to PI have been studied in subjects vaccinated with the PI fraction, in Brucellosis patients and normal controls.

INTRODUCTION

We have in recent years extracted numerous fractions from the cell wall of *B. melitensis* (Lacave, Asselineau Serre & Roux, 1969; Roux, Asselineau, Serre & Lacave 1967). Several of these fractions have been shown to possess immunogenic and protective properties and not to produce 'allergic' reactions in guinea-pigs and mice (Roux *et al.*, 1967; Lopez Merino, Asselineau, Serre, Roux, Bascoul & Lacave, 1976). One, namely fraction PI, has been used for several years for the vaccination of laboratory and agricultural workers considered 'at risk' (Roux, Asselineau, Serre & Lacave, 1970).

Correspondence: Dr Simone Bascoul, Laboratoire d'Immunologie, Institut de Biologie, Boulevard Henri IV, 34000 Montpellier, France.

Cellular immunity is know to play an important role in the immune response to facultative intracellular bacteria (MacKaness, 1969; North, 1973) and lymphocyte transformation is currently believed to correlate with delayed hypersensitivity (Mills & Harden, 1966).

We have therefore, in the present work, used fraction PI, together with another phenol-extracted peptidoglycan-containing fraction (4A) in a lymphocyte transformation test (LTT). Correlation between stimulation index (SI), humoral antibodies to *Brucella* and the cutaneous response to PI have been studied in subjects vaccinated with the PI fraction, in brucellosis patients and in normal controls.

LTT with fraction PI has been shown to be an useful tool for measuring the cellular immunity to *Brucella*.

MATERIALS AND METHODS

(1) *Antigen extraction*

As previously described in (Lacave *et al.*, 1969) fraction PI is extracted by Westphal's method from *Brucella melitensis* M15, a strain obtained in our laboratory from a case of human brucellosis.

Fig. 1 summarizes the extraction procedure: PI is the phenol-insoluble phase obtained after three

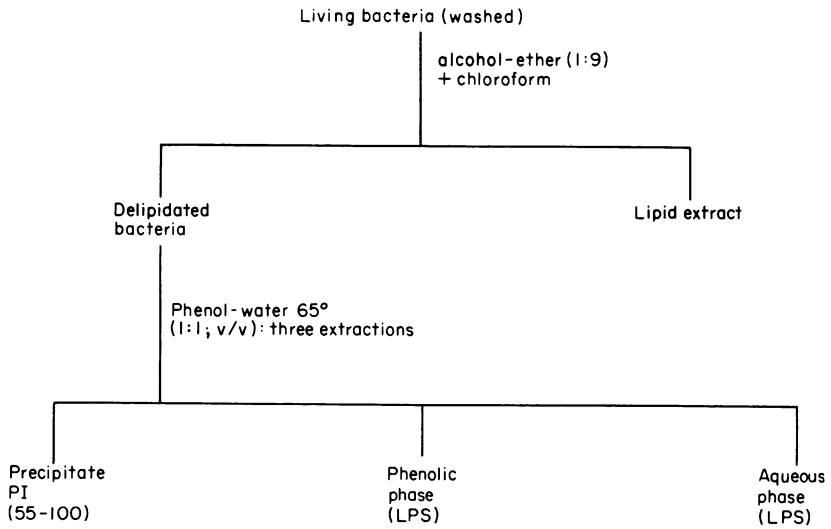

Figure 1. PI extraction from *B. melitensis*.

phenol/water extractions at 65°. It has been shown (Lacave *et al.*, 1969) to contain proteins, sugars and amino-sugars (mainly hexosamines).

Peptidoglycan is included in this fraction together with lipids and trace nucleic acids.

Fig. 2 shows that further purification of fraction PI by enzymatic treatment gives rise to several other fractions. Of these, fraction 4A has been chosen in this work because it confers a good protection in mice (Lopez-Merino *et al.*, 1976). Although the purification procedure eliminates about 90 per cent of PI material, fraction 4A remains a complex fraction; it still contains the peptidoglycan together with proteins, lipids, sugars and trace nucleic acids. Both fractions have been used as homogenized and tyndalized suspensions at concentrations ranging from 0·1–1·000 μg/ml.

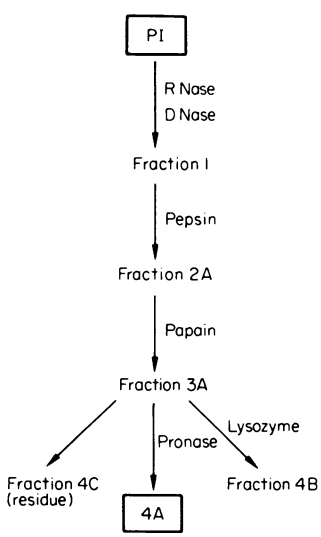

Figure 2. Enzymatic purification of fraction PI.

(2) *Forty-nine subjects have been studied*

These include the following. (a) Eleven unvaccinated controls: young adults without clinical history of a brucellosis-like syndrome and whose humoral and cutaneous tests to *Brucella* were negative. (b) Twenty patients including acute and chronic cases of brucellosis. (c) Eighteen vaccinated subjects received two injections of 0·5 mg fraction PI at a 1-month interval 1 week to 9 years prior to testing.

(3) *Lymphocyte transformation test*

Circulating lymphocytes separated on a Ficoll metrizoat gradient are washed and cultivated in M 199 + 20 per cent human AB serum in Falcon microtest culture plates (100·000 cells/well). PI and 4A fractions are used at concentrations of 0·1, 1, 10, 100 and 1000 μg/ml in triplicate wells.

Tritiated thymidine (CEA, specific activity 2 Ci/mM) 0·2 μCi/well is added 18 h prior to harvesting the cells on day 5.

The stimulation index (SI) is calculated by compar-

ing the incorporation by cells with and without the *Brucella* fractions

$$SI = \frac{\text{c.p.m. in test wells}}{\text{c.p.m. in control wells}}.$$

Viability tests are included together with controls with PHA (Difco) and Con A (Miles). The cells are harvested on day 3.

(4) *Humoral antibodies*

Humoral antibodies have been measured by Wright's agglutination test and by indirect immunofluorescence on ethanol fixed smears of *Brucella* (*B. suis* strain 1330) using a commercial FITC conjugated anti-human immunoglobulin serum diluted 1/100 (Institut Pasteur).

(5) *Cell-mediated immunity*

The sizes of cutaneous reactions have been measured 48 hours after intradermal injection of 0·1 ml of 1 μg/ml PI solution.

RESULTS

(1) Normal controls

No mitogenic activity of fraction PI or 4A for normal lymphocytes has been seen: SI ranges from 0·5 to 1·5. (mean = 1). At the concentrations used in this study fraction PI and 4A exert no cytotoxicity on human lymphocytes. (Cytotoxicity is however observed with PI concentrations of 10·000 μg/ml or more).

(2) Brucellosis patients

Fig. 3 shows the mean SI obtained in brucellosis patients using fraction PI and 4A at a concentration of 100 μg/ml. Stimulation by PI gives a mean SI (PI.SI) of 8·72, stimulation by 4A a mean SI (4A.SI) of only 1·71.

No significant difference between tests from acute or chronic cases of brucellosis has been found.

(3) Vaccinated subjects

The mean PI.SI (using 100 μg/ml) in our eighteen vaccinated subjects taken as a whole is 3·31, whilst the mean 4A.SI is only 1·5.

There is however a great variability in the PI.SI of vaccinated subjects since these range from 1–16·8.

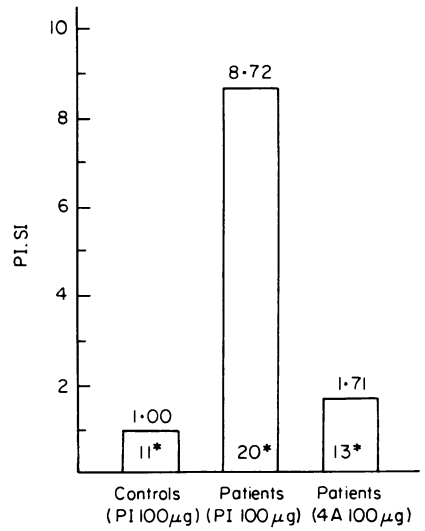

Figure 3. Mean stimulation index with PI and 4A in controls and in brucellosis patients. * = Number tested.

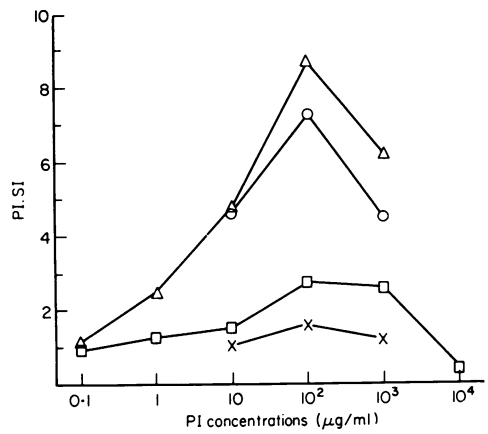

Figure 4. Relationship between PI concentration and the stimulation index. Each point represents the mean PI.SI of several subjects,: (△) brucellosis patients; (□) recently vaccinated subjects; (○) vaccinated subjects with a long established vaccination and a high PI.SI; (×) vaccinated subjects with a long-established vaccination but a low PI.SI.

This is not due to dose-response variability in different subjects since Fig. 4 illustrates the fact that 100 μg/ml seems to be the optimal stimulating concentration in all cases studied so far both for PI and for 4A.

On the other hand Fig. 5 shows that recently vaccinated subjects (vaccinated less than 6 months

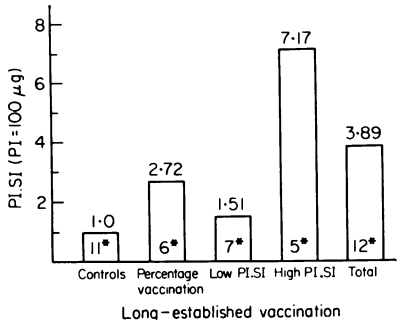

Figure 5. Mean stimulation index in vaccinated subjects with PI (100 μg). * = Number tested.

prior to testing) have a mean PI.SI of 2·72 while subjects vaccinated 6 months to 9 years prior to testing have a mean PI.SI of 3·89 with a clear-cut difference between two homogeneous groups: subjects with a low PI.SI (1·54) and subjects with a PI.SI very similar to that of brucellosis patients (7·17).

The latter have all been in close and repeated contact with *Brucella* in our laboratory while most of the former have never manipulated *Brucella* since vaccination.

(4) Kinetics of lymphocyte sensitization after vaccination with fraction PI

Fig. 6 shows the evolution of PI.SI in a subject tested from the 7th day to the 13th month after vaccination: LT tests, negative before vaccination, give positive SI as early as 7 days after PI inoculation while the second injection does not induce a significant rise of the PI.SI.

This may be considered as an illustration of a more general feature since in all cases studied so far the same pattern of response has been found.

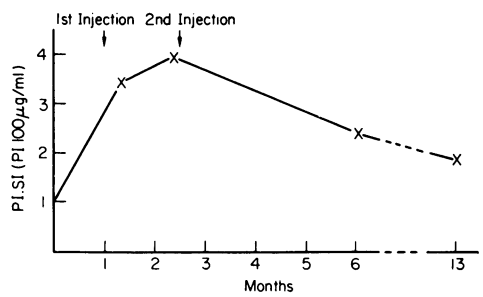

Figure 6. PI.SI evolution in a recently vaccinated subject.

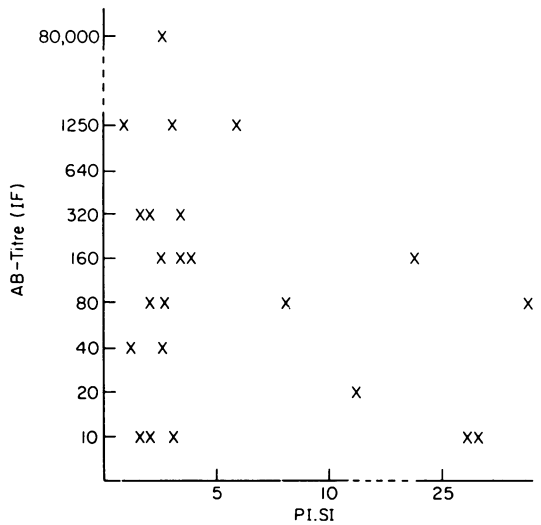

Figure 7. Correlation between stimulation index and antibody titres each cross represents the titre observed by the immunofluorescence test in an individual serum.

(5) Comparison between lymphocyte stimulation by fraction PI and the titre of humoral antibodies to *Brucella*

We have established this comparison in twenty-five subjects using the highly sensitive immunofluorescence test. Fig. 7 illustrates the absence of correlation between PI.SI and antibody titre. The calculated correlation coefficient is −0·115 and the regression lines calculated for SI f/antibody rate ($y = 0{\cdot}x + 8{\cdot}45$) and/or Ab rate f/SI ($x = -175{\cdot}55y + 4875{\cdot}20$) show that SI and the rate of circulating antibodies are two independant factors.

(6) Comparison between lymphocyte stimulation by PI and the cutaneous delayed reaction to PI

This has been studied in twenty-three subjects. The mean PI.SI is 7·84 in ID positive and 1·35 in ID negative subjects (these include the controls and three vaccinated subjects). Although the calculated correlation coefficient between PI.SI and the size of cutaneous reactions 48 h after intradermal inoculation of fraction PI is only 0·38 with regression lines of respectively $y = 1{\cdot}86 + 2{\cdot}15$ for SI f/mean diameter and $x = 0{\cdot}08y + 0{\cdot}53$ for mean diameter f/SI, this demonstrates a better correlation of PI.SI with delayed reaction to PI than with the titre of humoral antibodies to *Brucella*.

DISCUSSION

This is, to our knowledge, the first description of an *in vitro* stimulation of human lymphocytes by a purified fraction extracted from the cell wall of *B. melitensis*. Moreover, the use of such fractions for the comparative testing of patients and of vaccinated subjects has brought out the following points. (a) The two peptidoglycan-containing fractions studied in this work behave non-mitogenically towards normal human lymphocytes. Their behaviour towards murine lymphocytes is under study: preliminary results point to a discrepancy between human and murine mitogenicity, a phenomenon already observed for LPS (Andersson, Melchers, Galanos & Lüderitz, 1973), for other peptidoglycan-containing fractions and for the lipoprotein extracted from the outer membrane of *E. coli* (Nauciel, Fleck, Martin & Mock, 1973; Melchers, Braun & Galanos, 1975). (b) PI, currently used in our laboratory as a vaccinating agent for man and mice is shown to stimulate *in vitro* human lymphocytes sensitized either by a *Brucella* infection or by PI vaccination.

The stimulation index of brucellosis patients is however significantly higher than that of vaccinated subjects unless these are in close and repeated contact with *Brucella*. When such is the case PI.SI rises to rates similar to those of brucellosis patients although no clinical symptoms of brucellosis are seen. Of course, only long-term statistical epidemiologic studies will allow definite conclusions as to the efficiency of human vaccination with fraction PI. Our observations in laboratory workers nevertheless suggest that PI-vaccinated subjects are in fact well protected and that PI.SI could be a good tool for measuring their protection against *Brucella*. (c) Fraction PI can also be used in cutaneous tests of delayed hypersensitivity to *Brucella* and we have shown that it gives less allergic reactions and more reproducible results than does melitin (unpublished data).

A good correlation has been shown in the present study between cutaneous tests and PI.SI except in vaccinates. Our results suggest however a higher and earlier sensitivity of the LT test and indeed recently-vaccinated subjects whose PI.SI is significantly positive often display negative cutaneous tests both to PI and to melitin.

On the other hand, there is no correlation between the titre of humoral antibodies and PI.SI. Moreover, vaccination by fraction PI induces but low and transitory IgM antibody responses in man and the highly sensitive immunofluorescent test has shown no humoral anti-*Brucella* antibodies in several chronic brucellosis patients with a high PI.SI.

Taken together these results demonstrate the efficiency of PI.LT tests for the detection of cellular immunity to *Brucella* in man and add confirmatory data to previous experimental work on the predominant role of cellular rather than humoral immunity in protection against facultative intracellular bacteria (MacKanness, 1969). (d) Compared with fraction PI, fraction 4A which results from the purification of PI by enzymatic treatment is a poor stimulating agent for sensitized human lymphocytes *in vitro*. This suggests either that the more complex structure of fraction PI plays a role in lymphocyte activation *in vitro* or that, during the purification of fraction 4A (which includes the loss of 90 per cent of PI material), a component, responsible for the *in vitro* activation of sensitized lymphocytes, has been in part, lost. On the other hand, while it has not yet been used in man, fraction 4A has been shown to induce a good immunity to *Brucella* in mice (Lopez Merino *et al.*, 1976).

Further studies are in progress to test its effect on murine sensitized lymphocytes. This should help two elucidate whether or not protection by immunization and activation of sensitized lymphocytes are two separate functions elicited by two different constituents.

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RESULTS

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