In this study, we analyzed phagocytic cell function in 51 patients with active brucellosis and its relationship with different clinical, serological, and evolutionary variables. A control group was made up of 30 blood donors of similar geographic extraction, age, and sex, with no previous history of brucellosis or known exposure to the infection or specific antibodies. The investigations were carried out at the time of diagnosis, at the conclusion of treatment, and after 6 months of follow-up. Polymorphonuclear leukocyte adherence and nitroblue tetrazolium reduction in response to Brucella antigen were significantly increased in the patients at the time of diagnosis with respect to the control group. In contrast, chemotaxis in response to Brucella antigen and phagocytosis were significantly reduced in the patients with respect to the control group. The alterations in phagocytic cell function were greater in patients with bacteremia, with focal forms of the disease, or with a longer diagnostic delay. Most of these initial alterations tended to normalize with treatment, indicating their transient character.

Materials and Methods

Fifty-one patients with active brucellosis diagnosed in the Infectious Diseases Unit of the Regional Hospital in Málaga, Spain, between 1 January 1989 and 30 June 1990 were included in the study. Thirty-seven (72.5%) of the patients were male, and 14 (27.5%) were female, with a mean age of 37.2 years (range = 14 to 77).

All of the patients included in the study had a clinical picture compatible with brucellosis. In addition, they displayed one or more of the following characteristics: (i) isolation of Brucella organisms by means of culture from blood, body fluid (e.g., cerebrospinal or synovial fluid), or tissue biopsy; (ii) determination of specific antibodies at significant titers; and (iii) seroconversion in two matched samples 2 or 3 weeks apart.

The control group was composed of 30 voluntary blood donors with no exposure to Brucella spp. or history of brucellosis, in whom antibodies were not detected with the Bengal Rose test or by seroagglutination.

The blood cultures were processed with the usual technique (1, 27) with a BACTEC 730 system (Becton Dickinson), incubation being maintained for a period of 4 weeks or more. The presence of specific antibodies was investigated by means of the rose bengal test, seroagglutination, indirect immunofluorescence, and the Coombs anti-Brucella test according to previously described techniques (13, 15, 19, 21).

Once diagnosed, the patients were treated with doxycycline (100 mg/12 h per os for 45 days) and streptomycin sulfate (1 g/day intramuscularly for the first 21 days).

In cases of focal forms of infection, the doxycycline treatment was maintained for at least 2 months.

Isolation of mononuclear and polymorphonuclear leukocytes. Mononuclear cells were isolated from defibrinated blood by means of a density gradient according to the method of Boyum (6), washed, and resuspended in RPMI culture medium. The suspension was counted in a Neubauer chamber and adjusted to $3 \times 10^6$/ml.

The polymorphonuclear leukocytes were obtained by sedimentation of the erythrocytes with dextran from the buffy coat layer. Once isolated, the polymorphonuclear leukocytes were washed, and the concentration was likewise adjusted.

Phagocytic cell functions were studied by means of the tests described below.

Adherence test. The capacities of adherence to a smooth glass surface of the mononuclear and polymorphonuclear populations were evaluated separately, by means of the index of adhered cells in 10 ($\times 100$) fields according to Roy's technique (24), reducing the time of incubation to 30 min.

Assessment of chemotaxis. Assessment of chemotaxis was carried out with a modified Boyden technique (5), evaluating the number of cells in 10 fields ($\times 100$) passing through an 8-μm-pore-size filter after incubation for 2 h at 37°C. Culture medium was placed in the lower part of the chamber to assess chemokinesis. Active chemotaxis was stimulated nonspecifically with zymosan and specifically with Brucella antigen (suspension of Brucella abortus B19 inactivated with phenol from Biomerieux, Marcy l'Etoile, France, in a 50% solution with rabbit complement).

Assessment of phagocytosis. Assessment of phagocytosis
RESULTS

The duration of the clinical picture was 3.49 ± 2.61 days. The percentage of patients whose disease lasting less than 15 days was 28.1% (14/51). The results of patients who ingested Brucella melitensis were done by using a modified Leifert technique. Studying the NBT test, the percentage of nitroblue tetrazolium (NBT) was done according to the method of Park et al. (22), as modified by Regnery et al. (10), with and without stimulation by Brucella antigens. The conclusion reached was that the statistical significance was not increased by the end of the 6-month follow-up period, but had returned to normal. The adherence of mononuclear cells showed a similar pattern without statistically significant differences. Chemokinesis was also increased with respect to the control group. The results of the tests are presented in Table 2.

TABLE 2. Phagocytic cell function in controls and patients at diagnosis and its evolution during follow-up in patients

<table>
<thead>
<tr>
<th>Group</th>
<th>Chemokinasis</th>
<th>Chemotaxis with Brucella antigen</th>
<th>Adherence of:</th>
<th>% Phagocytosis of:</th>
<th>% NBT reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nonstimulated</td>
</tr>
<tr>
<td>Controls</td>
<td>45.37 ± 31.16</td>
<td>195.34 ± 89.91</td>
<td></td>
<td></td>
<td>18.51 ± 8.11</td>
</tr>
<tr>
<td>Patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pretreatment</td>
<td>93.35 ± 114.19</td>
<td>127.64 ± 97.56</td>
<td>120.52 ± 127.61</td>
<td>92.75 ± 76.97</td>
<td>78.82 ± 18.00</td>
</tr>
<tr>
<td>Posttreatment</td>
<td>154.07 ± 218.99</td>
<td>175.65 ± 97.72</td>
<td>79.18 ± 69.24</td>
<td>77.69 ± 87.24</td>
<td>86.81 ± 11.01</td>
</tr>
<tr>
<td>After 6 mo</td>
<td>101.62 ± 122.58</td>
<td>195.17 ± 121.51</td>
<td>98.78 ± 75.48</td>
<td>70.95 ± 45.91</td>
<td>85.96 ± 12.51</td>
</tr>
</tbody>
</table>

* Values are means ± standard deviations.
* * Number of cells passing through an 8-μm pore-size membrane in 10 fields (×100).
* * Number of cells adhered to a glass slide in 10 fields (×100).
* * Percent cells with phagocytosed particles.
* * Percent cells with formazan inside.
* * Result was significantly (P < 0.01) different from that in control.
* * Result was significantly (P < 0.05) different from that in control.
* * Result was significantly (P < 0.01) different from that pretreatment.
group at the time of diagnosis and at the conclusion of treatment, descending significantly at the 6-month follow-up, although not returning to normal. On the other hand, the chemotaxis stimulated by \textit{Brucella} antigen was significantly reduced ($P < 0.001$). It slowly and significantly increased throughout the follow-up period ($P < 0.05$) until it reached normal levels.

The phagocytosis of polymorphonuclear leukocytes and monocytes was significantly depressed in the patients at the time of diagnosis ($P < 0.001$ in both cases), rising after completion of the treatment. It did not reach normal values by the end of the follow-up period. We found no differences between patients and controls when we studied the NBT test in the absence of any stimulant. However, in the presence of \textit{Brucella} antigen, the reduction of NBT increased significantly ($P < 0.005$), remaining high at the end of the follow-up period.

As well as analyzing the possible differences in phagocytic cell function between patients and controls and the influence of treatment on the alterations found, we tried to relate the different immunological parameters studied to some variables of recognized importance in the prognosis of brucellosis. The variables analyzed were age, duration of the clinical picture, presence of focal forms, absolute number of leukocytes and lymphocytes, erythrocyte sedimentation rate, presence of bacteremia, and specific serological response. Because of the low number of patients with recidivism and therapeutic failure in our study, we were unable to evaluate the influence of immunological alterations on the unfavorable forms of evolution of the disease.

Tables 3 and 4 show the effects of bacteremia and the presence of focal forms of the disease on phagocytic cell function. Cells from patients with bacteremia presented less polymorphonuclear leukocyte adherence and chemokinesis than those from patients with a negative blood culture, although this was not statistically significant. On the other hand, NBT reduction was significantly higher in polymorphonuclear leukocytes obtained from patients with bacteremia ($P < 0.05$).

The adherence of polymorphonuclear leukocytes and mononuclear cells showed a marked tendency to decline in the patients with focal forms of the disease, although these data were not statistically significant. On the other hand, the chemotaxis in response to \textit{Brucella} antigen showed a certain tendency to rise in the focal forms, although this rise was also not statistically significant. As far as the duration of the clinical picture is concerned, there seemed to be a drop in the level of adherence of polymorphonuclear leukocytes in the patients in which the disease was diagnosed late, although this was not statistically significant. Nevertheless, the chemokinesis in cells from patients with a delayed diagnosis increased (Table 5), although the increase was also without statistical significance.

**DISCUSSION**

Brucellosis is a systemic infection which often follows a difficult course, with numerous relapses and focal complications despite being subjected to an adequate antibiotic treatment (2).

This peculiar form of disease evolution seems to be related to the location of the microorganism within the cells of the mononuclear-phagocytic lineage. In the interior of these cells, the microorganism is, to a certain extent, protected from the microbicidal action of antibiotics (11). Nonetheless, \textit{Brucella} organisms have been shown to be able to multiply within these cells, implying an ability to evade attack by the immune system. In the present study, we have analyzed the phagocytic cell function in active human brucellosis.

Our results imply that, in brucellosis, the immune response is initially adequate, and the adherence of polymorphonuclear leukocytes and monocytes increases, as does their chemokinesis.

The increase in the spontaneous mobility of polymorphonuclear leukocytes found in the patients with respect to the control group and its inhibition in the presence of \textit{Brucella} antigen seem to indicate that there is some factor associated with the microorganism which is capable of reducing the migratory capacity of the phagocytes. Furthermore, in patients with bacteremia, the chemokinetics of the polymorphonuclear leukocytes is reduced.

Since, in previous studies carried out by our group, we found no alteration of the complement system, as assessed by total hemolytic activity (data not shown), this variation in mobility does not appear to be attributable to this system.

Although we have not found any similar studies in the literature reviewed, our results are compatible with those of Birmingham et al. (4), who found several factors which either inhibited or activated migration. These factors resembled the lipopolysaccharide moieties found on the outer external membrane of \textit{B. abortus} (4).

**TABLE 3. Effects of bacteremia on different tests of phagocytic cell function**

<table>
<thead>
<tr>
<th>Bacteremia</th>
<th>Adherence of polymonuclear cells (mean ± SD)$^a$</th>
<th>Chemokinesis (mean ± SD)$^b$</th>
<th>NBT reduction with Brucella antigen (mean ± SD)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>88.50 ± 60.62</td>
<td>54.71 ± 71.91</td>
<td>45.28 ± 17.22</td>
</tr>
<tr>
<td>Absent</td>
<td>172.50 ± 173.39</td>
<td>87.10 ± 90.27</td>
<td>34.78 ± 11.40$^d$</td>
</tr>
</tbody>
</table>

$^a$ Number of polymonuclear cells adhered to a glass slide in ten fields ($\times 100$).

$^b$ Number of cells passing through an 8-µm-pore-size membrane in 10 fields ($\times 100$).

$^c$ Percent cells stimulated with \textit{Brucella} antigen with formazan inside.

$^d$ $P < 0.050$.

**TABLE 4. Pattern of phagocytic cell function in focal forms of the disease**

<table>
<thead>
<tr>
<th>Focal forms</th>
<th>Adherence of polymonuclear cells (mean ± SD)$^a$</th>
<th>Adherence of mononuclear cells (mean ± SD)$^b$</th>
<th>Chemotaxis with \textit{Brucella} antigen (mean ± SD)$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Present</td>
<td>85.63 ± 30.69</td>
<td>74.46 ± 84.56</td>
<td>174.90 ± 109.05</td>
</tr>
<tr>
<td>Absent</td>
<td>130.35 ± 140.96</td>
<td>97.40 ± 84.56</td>
<td>112.35 ± 89.94$^d$</td>
</tr>
</tbody>
</table>

$^a$ Number of polymonuclear cells adhered to a glass slide in 10 fields ($\times 100$).

$^b$ Number of mononuclear cells adhered to a glass slide in 10 fields ($\times 100$).

$^c$ Number of cells passing through an 8-µm-pore-size membrane in 10 fields ($\times 100$) against \textit{Brucella} antigen.

$^d$ Result was almost significantly ($P < 0.0064$) different from that in patients with focal forms.

**TABLE 5. Effects of diagnostic delay on phagocytic cell function**

<table>
<thead>
<tr>
<th>Duration of clinical picture (days)</th>
<th>Adherence of polymonuclear cells (mean ± SD)$^a$</th>
<th>Chemokinesis (mean ± SD)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;14</td>
<td>140.21 ± 96.58</td>
<td>60.83 ± 99.16</td>
</tr>
<tr>
<td>15–90</td>
<td>116.71 ± 141.44</td>
<td>92.20 ± 108.58</td>
</tr>
<tr>
<td>&gt;90</td>
<td>82.00 ± 122.07</td>
<td>199.25 ± 161.13</td>
</tr>
</tbody>
</table>

$^a$ Number of polymonuclear cells adhered to a glass slide in 10 fields ($\times 100$).

$^b$ Number of cells passing through an 8-µm-pore-size membrane in 10 fields ($\times 100$).
It is possible that the predominance of some factors over others influences the different levels of virulence of the different Brucella spp.

The predominance of the inhibition found in our study would be justified by the recognized virulence of B. melitensis, the only species isolated from our patients (3).

This factor or factors could also be responsible for the reduction in the phagocytic capacity of the polymorphonuclear leukocytes and monocytes seen in our study.

The reduction in phagocytosis found by us contrasts with the findings of Young et al. (28) and Gallego et al. (17), who did not find any alterations in phagocytosis in the polymorphonuclear leukocytes from goats or humans in the presence of Brucella organisms. Nevertheless, our results are in complete agreement with those of Burgaleta et al. (7). The explanation for this apparent discrepancy could be derived from the fact that both our study and that of Burgaleta et al. were carried out with patients with active disease, whereas those of Young and Gallego et al. refer to animals and healthy humans.

In the present study, we have only explored the intracellular microbicidal capacity by means of the oxidative pathway. In the absence of stimulants, the microbicidal function in the patients did not differ from that of the controls, which shows that there are no apparent alterations in the oxidative pathway of these cells. Likewise, this pathway is stimulated in the presence of Brucella antigen, with a significant increase in the reduction of NBT in cells from the patients.

Our findings agree with those of Canning et al. (9), who were able to demonstrate that several different fractions of B. abortus do not inhibit the production of superoxide anion in bovine polymorphonuclear leukocytes, nevertheless inhibiting the microbicidal action by myeloperoxidase. The same group related the inhibition of iodination of the proteins by myeloperoxidase to the presence of low-molecular-weight fractions obtained from Brucella spp. The substances responsible for this inhibition were later identified as 5'-GMP and adenine (8).

More recently, Orduña et al. have been able to verify the inhibition of the degranulation and myeloperoxidase activity in human polymorphonuclear leukocytes in the presence of B. melitensis (22). It could therefore be suggested that, in patients with active brucellosis, the respiratory burst is adequately initiated, generating \( \text{H}_2\text{O}_2 \), but that the degranulation of the primary granules to liberate myeloperoxidase is partially or totally inhibited by the microorganism.

This event conditions the intracellular survival of the aggressor agent, explaining the persistence of the infection and its tendency to recidivism.

In our study, we have been able to verify that the adherence and chemokinesis of the polymorphonuclear leukocytes are depressed in the presence of bacteremia, a finding not previously reported and which could partly explain the great tendency of B. melitensis to surpass the defense system and enter the blood stream. Furthermore, the patients with greater diagnostic delay and focal forms of the disease seem to show greater alterations in measures of phagocytic cell function.

We have found no relationship between the serological response and phagocytic cell function, which appears to corroborate the previous impression that the specific humoral response plays little importance in the eradication of this infection (25).

From our study, it can be concluded that B. melitensis is responsible for alterations in the chemotaxis and phagocytosis of polymorphonuclear leukocytes and mononuclear cells and that these alterations revert with adequate treatment, which demonstrates their dependence on Brucella infection.

Further studies are needed in order to evaluate the relationship between the alterations in phagocytic cell function and the cellular response and the subsequent release of lymphokines, which might help clarify the torpid and relapsing character of this frequent infection.

ACKNOWLEDGMENTS

We gratefully acknowledge the expert technical assistance of A. Chamorro and the nurses of the Internal Medicine Department and the Infectious Diseases Unit. This work was supported by grant 89/0305 from FISS.

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