Stable Hyperzinchemic Condition in Patients with Behcet's Syndrome. Lack of Correlation with *In Vitro* Cellular Immune Response Capacity

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Summary

We have observed a significant increase of zinc concentration in plasma and erythrocytes in a group of Italian Behcet patients, evidencing a rare hyperzinchemic condition associated with the disease. The increased zinc level observed does not show any relation to the presence of therapy or with the impairment of the blastogenic response to HSV1, a specific cellular defect in Behcet disease, confirmed in the present study. The possible biological meaning of these observations is briefly discussed.

Keywords: Zinc, hyperzinchemia, Behcet's disease, cellular immune response.

Introduction

Behcet's disease (BD) is a multisystem disorder characterized by a multifocal vasculitis which has its clinical hallmarks in: (a) recurrent aphtous ulcerations of oral mucous membranes coming in crops lasting one to two weeks; (b) skin lesions, including erithema nodosum, superficial thrombophlebitis, folliculitis and acne-like lesions, and cutaneous hypersensitivity; (c) ocular symptoms (Figure 1) such as iridocyclitis, featuring transient hypopyon iritis and

Figure 1. Characteristic ocular lesions of BD: iridocyclitis and episcleritis.

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posterior synechiae, chorioretinitis, and their sequels; (d) genital ulcers or painful punched-out lesions (1). Minor criteria include arthritis, intestinal ulcerations, epididymitis, thrombophlebitis and neuropsychiatric symptoms. The complete type of BD requires the four main symptoms to appear some time during the clinical course, whereas the incomplete type requires the three major criteria to appear some of the time, or that recurrent hypopyon iritis or retinitis is present, accompained by one other major criterion (1).

Although the etiology of the disease is still unknown and both viral and autoimmune hypotheses have been put forward, several findings suggest a cell-mediated immune pathogenesis. The immunological derangement of the disease is evidenced by the presence of immune complexes in serum (2), the imbalance of the T-cell subsets (3) and the specific impairment of the lymphoproliferative response to Herpes Simplex Virus type 1 (4). Furthermore, HLA B51 antigen has been shown to be strongly associated with the ocular type of the disease (5, 6), suggesting the involvement of immunogenetic factors.

Finally, neutrophil activation is thought to be of pathogenetic relevance, particularly in the ocular type of the disease (7). Zinc represents one of the most important regulatory trace elements in the cellular immune response mediated by lymphocytes activation (8, 9); the importance of this oligoelement in eliciting

a normal immune response is confirmed by several observations of cellular impairment in hypozinchemic conditions such as restriction of dietary zinc, acrodermatitis enteropathica or chronic renal insufficiency in patients under haemodialitic treatment (10–13).

In the attempt to evaluate a possible involvement of zinc in the immunopathogenesis of Behcet's disease, we have analyzed zinc concentrations in plasma, serum and erythrocytes in a group of Italian Behcet patients. The patients were also tested for the lymphoprolipherative response to mitogens (Phytohaemagglutinin, Pokeweed Mitogen), alloantigens (by Mixed Lymphocytes Reaction) and HSV1.

Materials and Methods

Population

The study is based upon 11 Italian patients all affected with the ocular type of BD and diagnosed as complete (n = 8) and incomplete (n = 3) according to the clinical criteria proposed by the Behcet's Disease Research Comittee of the Japanese Ministry of Health · and Welfare (1). It was possible to reexamine, 3 to 5 months later, 5 of the 11 BD patients. At the time of the study 4 BD patients were under therapy (all with steroids). Blood samples were also obtained from 24 volunteer healthy subjects.

Zinc concentration

Plasma, serum and erythrocyte zinc concentration was determined by flame atomic absorption spectrophotometry (Perkin Elmer mod. 1100). Venous blood samples were drawn into disposable plastic zinc-free syringes and transferred into plastic test tubes with zinc-free heparin for plasma and red blood cell separation.

Blood samples were separated within four hours of collection and plasma and sera showing haemolysis were discarded. The frozen sera and plasma were analyzed after 1:10 dilution of the sample with deionized water. For erythrocytic zinc evaluation 0.5 ml of centrifuged red blood cells for each sample were haemolyzed with cold deionized water to reach a haemolysate dilution of 1:100. The zinc concentration was determined using an analytical calibration curve and expressed as µmol/L; the use of the background corrector was necessary. The merits of the analytical procedures were the following: 1) relative standard deviation: 3.5% (1.5 µmol/L), maximum 5% at the lowest concentrations observed and minimum 1% at the highest concentrations observed); 2) limit of detection: 0.1 µmol/L (95%); 3) recovery: 94–101%.

Blastogenic response

Peripheral blood lymphocytes were separated in Lym-

phoprep (Nycomed AS, Norway), washed in PBS and resuspended at a concentration of 1×10^6/ml in Iscove medium (Gibco) supplemented with 10% inactivated AB pool sera. Lymphocyte cultures were made in 3072 Falcon microtiter plates using 0.1 ml of lymphocytic suspension. For mitogenic responses, preliminary experiments established the optimal range of concentrations for Phytohaemagglutinin P (Difco) and Pokeweed Mitogen (Gibco). For MLR, lymphocytes from 10 healthy subjects were inactivated by irradiation (4500 rads), pooled and used as stimulating cells; 1×10^5 responding lymphocytes and the same number of stimulating cells were cultured together in 0.2 ml medium.

The specific lymphoproliferative response to HSV1 was assessed by adding 0.1 ml of medium containing 45 mg/L of virus-purified proteins (14). Control cultures were prepared in the presence of an equal amount of cellular proteins obtained from the Hep-2 monolayer cell line in which virus is routinely grown. All cultures were performed in triplicate. Eighteen hours before harvesting, 1 µCi ^{3}H-thymidine (Amersham-England, spec. act.: 2 Ci/mmol) was added to each well. The results were expressed in stimulation indices (S.I. = cpm stimulated cultures/cpm unstimulated cultures).

Statistical analysis was performed using the Mann-Whitney U test, one-tailed or two-tailed as appropriate, and linear regression analysis.

Results

Zinc concentration in BD patients and healthy controls are reported in Table 1. Statistically significant increases were observed in BD patients, compared to controls, in plasma (p < 0.05) and erythrocytes (p < 0.02) (two-tailed Mann-Whitney U test) while serum didn't reach statistical significance. A further analysis performed on erythrocytes several months later, confirmed the stability of the zinc levels in pa-

Table 1. Plasma, serum and erythrocytes zinc concentration in Behcet patients and healthy controls. (Values are given in µmol/L)

	Behcet patients		Healthy controls		
	N	Mean ± SD Values range	N	Mean ± SD Values range	p
Plasma range	11	28.7 ± 12.8 (14.2–49.8)	24	16.2 ± 3.7 (9.3–23.8)	< .05
Serum range	5	20.2 ± 4.0 (16.4–26.0)	17	17.1 ± 3.1 (12.6–23.0)	NS
Erythrocytes range	11	179.4 ± 30.3 (115.0–212.9)	13	140.4 ± 29.4 (94.5–188.1)	<.02

Table 2. Erythrocyte zinc level of 5 Behcet patients observed at different times. (Values are given in μmol/L)

	I sample	II sample
Patient 1	209.2	192.6
Patient 2	156.0	168.1
Patient 3	196.5	201.2
Patient 4	156.4	162.0
Patient 5	212.9	190.6

tients (Table 2). The comparison between serum and plasma zinc levels within the two groups didn't show any significant difference; the slight differences observed could be merely due to the smaller size of the sample.

The patients' lymphoprolipherative responses to PHA, PWM, and alloantigens do not significantly differ from those observed in healthy controls (see Table 3); on the other hand, we were able to confirm the reported (15–17) impairment of the blastogenic response to the HSV1 antigen ($p < 0.001$; one-tailed Mann-Whitney U test) (Table 3). Blastogenic responses obtained in autologous and in AB pool serum didn't show statistically significant differences (data not shown). No correlation was found in BD patients between zinc concentration and the impaired response to HSV1 antigen ($r = .0219$ and $p = $ n.s.). The patients under corticosteroid therapy didn't show levels of zinc statistically different from those of untreated patients.

Table 3. Responses to PHA, PWM, MLR and HSV1 in Behcet patients and healthy controls expressed as stimulation indices. (Values are given in S. I.)

	Behcet patients		Healthy controls		
	N	Mean ± SD Values range	N	Mean ± SD Values range	P
PHA	11	160.17 ± 92.3 (55.30–311.30)	9	141.84 ± 123.89 (51.37–426.19)	NS
PWM	11	46.57 ± 40.88 (5.03–144.18)	9	58.92 ± 41.89 (12.03–137.30)	NS
MLR	11	30.56 ± 26.64 (4.49–90.67)	9	32.14 ± 20.66 (10.06–61.09)	NS
HSV1	11	3.96 ± 4.18 (1.09–15.41)	17	21.29 ± 13.68 (4.00–52.39)	< .001

Discussion

Zinc is one of the most important regulatory oligoelements in cellular immune response, effecting mainly T-lymphocytes, but also B-lymphocytes and phagocytes. The impairment of cellular response as a consequence of low zinc levels and the restoring of normal cellular immune capacity following dietary treatments has been reported in various studies, confirming the role of this element in the immune system regulation (10, 13). On the other hand, very little is known about the biological effect of increased zinc levels; up to now, sporadic observations such as familial hyperzinchemia (18) have been the only available data, with no investigations of possible effects on the cellular response.

In the present study we have observed a significant increase of zinc levels in plasma and erythrocytes of patients affected by BD. Our reference ranges, for serum and plasma, resulted higher than those generally reported (19), however these data are in agreement with those previously found in our population (13). Two out of the five patients examined for serum zinc concentration showed levels above the normal range; however the results didn't reach statistical significance, probably due to the small size of the sample.

The finding of high intraerythrocytic zinc levels suggests a stable hyperzinchemic condition in these patients, because of the long life span of erythrocytes (about 120 days). High zinc levels were also confirmed in further analyses performed at different times, thus excluding a temporary imbalance.

Inflammatory processes seem to influence serum zinc levels. In fact, in many chronic inflammatory diseases, such as Rheumatoid Arthritis, low serum zinc level is a common finding (20) and it is worth noticing that the fall in zinc concentration correlates with ESR, a good indicator of the precence of a phlogistic disease. Zinc seems to be lowered in serum because of its uptake in the inflamed tissues where interleukins may induce the increased synthesis of metal-binding proteins which serve a cellular protective function against free oxygen radicals (20). Surprisingly, in BD, a phlogistic disease considered by many authors to be connective tissue disorder just like RA, there was found not a decrease in zinc level but a significant increase.

Further investigation is needed in order to explain these data; however, it is tempting to speculate that this quite specific imbalance in BD could play an etiopathogenetic role. We speculate that zinc is increased because it cannot be utilized in BD patients in modulating the immune response and this incapability is reflected in the chronic tissue damage.

In order to support this hypothesis the origin of zinc imbalance should be tested by examination of cell lines from BD patients. Furthermore, it could be helpful to investigate the increase in the zinc intracellular level in BD neutrophils, which in our opinion are the most promising candidates for the pathogenesis of the tissue damage in the ocular type of BD (7).

Acknowledgements

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