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Mycobacterium leprae infection and serum lactoferrin levels

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Summary Serum lactoferrin level, using competitive enzyme linked immunosorbent assay (ELISA) method, was estimated in 298 leprosy patients admitted into the hospital and attending the out-patient department of the Schieffelin Leprosy Research and Training Center. Serum from an equal number of non-leprosy individuals served as control. Mean (SD) of serum lactoferrin in non-leprosy individuals was 0·277 (0·092) μg/ml while in leprosy patients it was 0·494 (0·394) μg/ml, the difference being significant ($P = 0{\cdot}0001$). Serum lactoferrin levels were not significantly associated with type 2 reactions ($P = 0{\cdot}613$). Serum lactoferrin was significantly associated with age ($P = 0{\cdot}006$), duration of the disease ($P = 0{\cdot}0001$), DDS monotherapy ($P = 0{\cdot}007$), deformity ($P = 0{\cdot}005$), average bacterial index (BI) ($P = 0{\cdot}01$) and smear positivity ($P = 0{\cdot}0001$), orbicularis oculi weakness ($P = 0{\cdot}001$), lagophthalmos ($P = 0{\cdot}002$), corneal opacity ($P = 0{\cdot}001$) and cataract ($P = 0{\cdot}004$) in simple regression analysis. All these variables, with the exception of smear positivity ($P = 0{\cdot}019$), lost their significance ($P > 0{\cdot}05$) when analysed using multiple regression. Serum lactoferrin showed poor association with type 1 ($P = 0{\cdot}286$) and type II reactions ($P = 0{\cdot}613$) and iridocyclitis ($P = 0{\cdot}207$). We conclude that serum lactoferrin is strongly and inversely associated with increasing BI but does not show significant association with type 2 reactions.

Introduction

Lactoferrin is a glycoprotein found in the exocrine secretions of mammals and is also released from neutrophil granules during inflammation. Receptors for lactoferrin are found in monocytes, lymphocytes, neutrophils, intestinal tissue and on certain bacteria. Several biological roles have been attributed to lactoferrin which include antibacterial, antiviral,

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antifungal, anti-inflammatory, antioxidant and immunomodulatory activities.[1] Levels of serum and plasma lactoferrin have been investigated in several diseases and in a few normal individuals.[2–10] Serum lactoferrin level in lepromatous leprosy has been estimated in an earlier study. Raised levels of serum lactoferrin in ENL reactions suggested a role of this glycoprotein in being a possible predictor of ENL reactions.[10] This study, however, was limited by a small sample size and other variables that might influence lactoferrin levels in these patients were not investigated. We did a cross-sectional study in which lactoferrin levels in the serum and tears of a large number of leprosy patients and an equal number of non-leprosy, healthy controls were estimated. We report on the associations found between serum lactoferrin levels and various demographic, leprosy and ocular characteristics in these leprosy patients and controls.

Materials and methods

Leprosy patients attending the outpatient department and admitted in the wards of the Schieffelin Leprosy Research and Training Center, Karigiri, categorized into specified groups were invited to participate in the study. The stratification included 1) smear negative leprosy patients who were newly diagnosed, under treatment and released from treatment (RFT), 2) smear positive patients who were newly diagnosed, under treatment and RFT, 3) patients with past and present type 1 reaction and type 2 reaction and 4) patients with eye complications related to leprosy like lagophthalmos and iridocyclitis. An equal number of persons who did not have leprosy but belonging to the same socioeconomic status (SES) served as controls. These were selected as much as possible to match the age and sex of patients. Individuals with obvious ocular abnormalities or known systemic diseases were excluded from the control group. After informed consent was obtained, each of the participating patients and controls was asked to complete a demography questionnaire. The patients were then assessed for leprosy characteristics that included type of leprosy categorized according to the Ridley and Jopling[11] classification and the WHO treatment classification, the approximate duration of the disease, skin smear positivity for acid fast bacilli, deformity grading according to the WHO classification, presence or absence of type 1 reaction or type 2 reaction, treatment status, relapse, presence or absence of face patch, extra clofazimine therapy and steroid therapy. Face patches were defined as either a hypo-pigmented patch or an erythematous patch occurring over the eyelids or over the malar area. The malar area was defined as an area 2 cm on either side of an imaginary line drawn from the lateral canthus of the eye to the tragus. This was followed by an ocular examination that included specifically looking for madarosis, orbicularis oculi weakness, lagophthalmos, ectropion, entropion, corneal opacity, features of past or present iridocyclitis, naso-lacrimal duct patency and cataract.

Blood (5 ml) was collected from all patients and controls by venous puncture and the serum separated by centrifugation. Tears were collected from the right eye using the glass capillary method after stimulation with a strong beam of light. These samples were stored at -20°C till use. Lactoferrin in serum and tear fluid was quantified by competitive enzyme linked immunosorbent assay (ELISA) method as used earlier by Kijlstra *et al.*,[12] with minor modifications. Briefly, the competitive binding of anti-human lactoferrin to free lactoferrin in serum against the micro plate bound lactoferrin was measured and the quantity calculated with the help of a standard graph. All standards and unknown samples were estimated in triplicates. Wells except blanks were coated with 100 μl of 0·05 μg/ml human milk lactoferrin

(Sigma) diluted in coating buffer for 1 h at 37°C. Excess lactoferrin was washed 3 times with 0·1% PBST. Standards were included in each plate with serially diluted human milk lactoferrin ranging from 0·1 μg/ml to 0·0015625 μg/ml. In test wells, 100 μl neat and 1:10 dilution of serum or tears at four different dilutions ($1:10^4$ to $1:10^7$) were added. Dilutions were made with incubation buffer (3% BSA in 0·1% PBST). To this, 100 μl of 1:2000 of rabbit anti human lactoferrin antibody (DAKO) prepared in 0·1% PBST was added and incubated at room temperature on shaker for one hour to facilitate competitive binding. Plates were washed 3 times with 0·1% PBST. Goat anti-rabbit (HRP) conjugate (100 μl; Dako) diluted 1:2000 with 0·1% PBST was added and incubated at room temperature for 1 h. It was then washed 3 times with 0·1% PBST and 100 μl of freshly prepared substrate ortho-phenylene diamine (Sigma) with 30% H_2O_2 was added to each well and the micro plate left in darkness at room temperature for 30 min. The reaction was stopped with 100 μl of 0·5 mol/l sulphuric acid and the optical density read at 490 nm. The standard curve drawn with known concentrations of human milk lactoferrin was used to plot the levels of lactoferrin in samples. Statistical analysis was done with the help of Microsoft Excel, Epicalc 2000 and Stata 7.0. Chi square analysis categorizing serum lactoferrin into less than 1 mg/ml and more than or equal to 1 mg/ml, simple regression and multiple regression using several models with continuous as well as categorized values were carried out.

Results

Two hundred and ninety eight leprosy patients and an equal number of controls agreed to participate in the study. There were 230 males and 68 females among patients, and the controls had 226 males and 72 females. Age of patients ranged from 15 years to 79 years with a mean (SD) of 42·9 (15·4) years. Age of controls ranged from 15 years to 79 years with a mean (SD) of 42·6 (15·4) years. There was no significant difference in the age or sex of both groups. Serum lactoferrin levels ranged from 0·06 μg/ml to 0·54 μg/ml with a mean (SD) of 0·277 (0·092) μg/ml in non-leprosy controls and from 0·02 μg/ml to 1·95 μg/ml with a mean (SD) of 0·494 (0·394) μg/ml in patients. The difference in serum lactoferrin levels between these two groups was significant ($P = 0·0001$). Serum lactoferrin increased by 0·0035 mg/ml for every 10 years increase in age among the non-leprosy controls, but this association was not significant ($P = 0·309$).

In the leprosy group, there were three tuberculoid tuberculoid (TT), 77 borderline tuberculoid (BT), two borderline borderline (BB), 104 borderline lepromatous (BL), 94 lepromatous leprosy (LL) and five pure neuritic leprosy patients (PNL). A total of 212 belonged to the multibacillary (MB) group and 86 to the paucibacillary (PB) group. The approximate duration of disease ranged from 1 year to 51 years with a mean (SD) of 11·27 (13·3) years. Five (15%) were relapsed patients, 34 patients had had only dapsone mono-therapy (DDS), 166 had only multidrug therapy (MDT), 44 had both DDS and MDT and 54 newly diagnosed patients had yet to start on anti-leprosy therapy. Twelve patients had classical face patches over the malar area, 77 patients had patches anywhere in the face and 221 patients were free of any face patch. In 52 patients there was a history of RR and 49 patients had signs of RR at the time of examination. Forty-six patients had history of type 2 reactions and 28 had type 2 reaction at the time of sample collection. Fifty-four patients had had extra clofazamine and 180 oral steroids during the course of their disease. Hundred and forty-two patients had grade 2 deformity in one or more limbs, 46 had grade 2 deformity in all

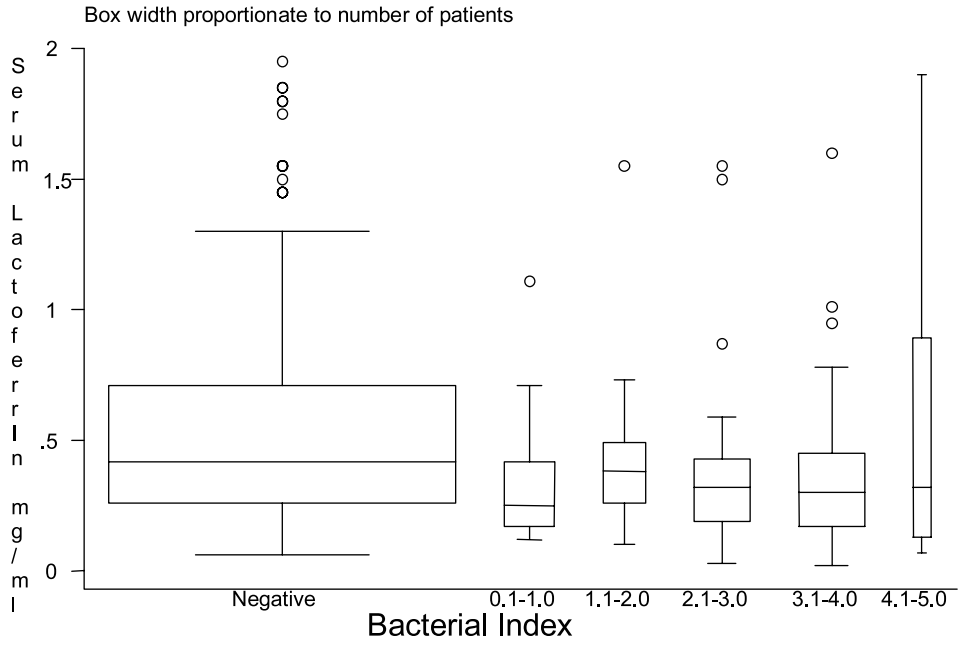

Figure 1. Box plot showing the spread of serum lactoferrin values in leprosy patients with varying Bacterial Index (BI).

the limbs and 84 had no deformity in any of the limbs. The average BI for acid-fast bacilli in the routine skin smears ranged from 0 to 4·5 with a mean (SD) of 1·38 (1·65) at the time of diagnosis of leprosy. One hundred and forty-three patients were smear negative when they were diagnosed with leprosy. The highest BI at any one site ranged from 0 to 6 with a mean (SD) of 1·32 (1·80) in patients at diagnosis. The average BI ranged from 0 to 4·5 with a mean (SD) of 0·98 (1·44) at the time of sample collection with 178 patients showing smear negativity. The highest BI at any one site ranged from 0 to 6 with a mean (SD) of 1·32 (1·79) in patients at the time of sample collection. The serum lactoferrin distributions by different bacterial indices are shown in Figure 1. Thirty-nine patients had madarosis, 69 had orbicularis oculi weakness, 66 had lagophthalmos, 18 had ectropion, three had entropion, 27 had signs of past iridocyclitis, nine had iridocyclitis, 57 had corneal opacity, 96 had cataract and one patient with blocked naso-lacrimal ducts.

Results of simple linear regression analysis on serum lactoferrin levels with demographic, leprosy and ocular variables are given in Table 1. Multiple linear regression between serum lactoferrin levels and all the variables of leprosy patients that showed significant associations in the simple linear regression analysis is given in Table 2.

Discussion

Serum and plasma lactoferrin levels have been investigated in several conditions, including sickle cell disease,[2] surgical stress,[3] rheumatoid arthritis, systemic lupus erythematosis,[4] cystic fibrosis,[5] psoriasis,[6] and neutropenia.[7] Contrary to expectations, a literature search

Table 1. Simple regression of serum lactoferrin with demographic, ocular and leprosy characteristics among leprosy patients

Variable	Coefficient	SE	95% confidence intervals		*P*-value
Age	0·00406	0·00147	0·00116	0·00696	0·006
Sex	−0·07210	0·05439	−0·17913	0·03494	0·186
Occupation	0·06679	0·04897	−0·02958	0·16317	0·174
Disease duration	0·18653	0·04917	0·08976	0·28330	< 0·00001
Relapse	0·11159	0·10451	−0·09408	0·31726	0·286
DDS monotherapy	0·14094	0·05143	0·03973	0·24215	0·007
MDT	0·02248	0·05017	−0·07624	0·12121	0·654
Face patch	−0·04168	0·05224	−0·14449	0·06113	0·426
Past type1 reaction	−0·01047	0·06031	−0·12917	0·10823	0·862
Present type1 reaction	−0·06588	0·06164	−0·18719	0·05543	0·286
Past type2 reaction	−0·04178	0·06332	−0·16639	0·08283	0·510
Present type2 reaction	0·03970	0·07843	−0·11464	0·19405	0·613
Oral steroids	−0·07120	0·04663	−0·16296	0·02056	0·123
Extra B663	−0·05241	0·05935	−0·16922	0·06439	0·378
Deformity	0·12791	0·04523	0·03889	0·21692	0·005
Average BI	−0·04105	0·01579	−0·07213	−0·00998	0·010
Smear positivity	0·16899	0·04563	0·07919	0·25880	0·001
Madarosis	0·07591	0·06773	−0·05739	0·20921	0·263
Orbicularis oculi weakness	0·18128	0·05324	0·07651	0·28605	0·001
Lagophthalmos	0·16298	0·05431	0·05609	0.26986	0·002
Ectropion	0·30495	0·09445	0·11908	0·49083	0·001
Entropion	0·32929	0·22852	−0·12044	0·77901	0·151
Past iridocyclitis	0·08314	0·07960	−0·07353	0·23980	0·297
Present iridocyclitis	0·16885	0·13340	−0·09369	0·43139	0·207
Corneal opacity	0·17529	0·05730	0·06251	0·28807	0·002
Cataract	0·13913	0·04832	0·04404	0·23421	0·004
Tear lactoferrin	0·00519	0·00317	−0·00104	0·01142	0·102

demonstrated a paucity of documentation on normal serum lactoferrin levels. Normal serum lactoferrin levels with mean (SD) 0·265 (0·21) μg/ml[8] and 0·237 (0·155) μg/ml[5] were found in two earlier studies. Our control group, which consisted mostly of people from a predominantly rural area in South India, shows a mean serum lactoferrin level of 0·277 μg/ml, which was similar to what was found in these two studies. However, this level is much lower than

Table 2. Multiple regression of serum lactoferrin with variables of leprosy patients that showed *P*-values of < 0·05 in the simple regression

Variable	Coefficient	SE	95% confidence intervals		*P*-value
Age	−0·00001	0·00199	−0·00393	0·0039	0·995
Duration of disease	0·00510	0·00310	−0·00140	0·01159	0·124
DDS monotherapy	−0·08604	0·09126	−0·26567	0·09359	0·347
Deformity	0·04436	0·05130	−0·05662	0·14534	0·388
Smear positivity	0·11278	0·04767	0·01895	0·20660	0·019
Orbicularis oculi weakness	0·39406	0·22282	−0·04452	0·83263	0·078
Lagophthalmos	−0·34993	0·22726	−0·79724	0·09738	0·125
Ectropion	0·11687	0·11401	−0·10754	0·34128	0·306
Corneal opacity	0·06801	0·06431	−0·05856	0·19458	0·291
Cataract	−0·00364	0·06600	−0·13354	0·12625	0·955

0·99 μg/ml, the mean level of lactoferrin found in the serum of normal controls in an earlier study. A study that looked at plasma lactoferrin levels found males and post-menopausal women to have a higher level than pre-menopausal females.[9] However, they found serum and plasma levels to be inconsistently elevated in all the groups studied. In our study the serum lactoferrin levels were identical in both sexes. We also did not find any correlation between age and serum lactoferrin levels in normal subjects.

Simple regression analysis performing ordinary least squares regression of the variable serum lactoferrin on single predictor variables like age, duration of disease, BI, DDS monotherapy, deformity, orbicularis oculi weakness, lagophthalmos, ectropion and cataract showed significant associations ($P < 0{\cdot}05$). When multiple regression was done adjusting for these predictor variables, only the association with BI remained significant ($P > 0{\cdot}019$). It is therefore clear that serum lactoferrin levels are at their highest levels in patients who are either negative for acid fast bacilli or have small quantities of bacilli, probably demonstrating the role of cell mediated inflammatory process and neutrophil degranulation in containing the bacillary multiplication. The reduced level of lactoferrin in patients with high bacterial content could be due to the generalized impairment of cell-mediated immunity seen among multibacillary patients. Anti-65kD antibody which cross-reacts with lactoferrin may also play a role in lowering free lactoferrin levels in the serum.[13]

An earlier study on leprosy patients found a weak positive correlation between increased levels of serum lactoferrin and the bacterial levels of *Mycobacterium leprae* in the body.[10] It also found elevated levels in those patients having type 2 reactions when compared with those who did not have these reactions. This study was focused on 38 lepromatous leprosy patients and the main objective was to see if serum lactoferrin was associated with ENL reactions. We did not find an association of raised serum lactoferrin levels with type 2 reaction, but instead found a significant inverse correlation with BI. This association remained significant on multiple regression analysis. These contradictory findings could been the result of our larger sample size (more than twice the number of lepromatous leprosy patients than were used in the previous study), our ability to analyse variables other than ENL that were likely to alter serum lactoferrin levels and because we ran a stepwise multiple regression on variables that were found to be significant in simple linear regression. We therefore were able to demonstrate more convincingly that it was the amount of *Mycobacterium leprae* in the body that was a significant variable altering serum lactoferrin levels while the ENL reactions were confounders.

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