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A Family Study of Leprosy: Subcutaneous Amyloid Deposits and Humoral Immune Responses[1]

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The many immunological abnormalities of leprosy have been reviewed ([14, 42]). However, both their frequency and the question of their preferential association with either one of the polar forms of the disease are under debate. For example, the reported prevalence of autoantibodies varies from 12% ([29]) to 58% ([25]) for rheumatoid factor, 0% ([29]) to 29% ([6]) for antinuclear antibodies, and 0% ([44]) to 48% ([5]) for thyroglobulin antibodies. The differences may in part be racial and in part due to varying representation of lepromatous and tuberculoid leprosy in the groups studied since it is generally ([29, 37]), though not universally ([31]), believed that autoantibodies are more frequent in the lepromatous form of the disease. Similar differences of opinion exist in regard to a well-recognized complication of leprosy, secondary amyloidosis, which has been claimed to have ([27]) or not to have ([8]) a close association with lepromatous leprosy.

Genetic studies have also suggested that there may be an association between HLA antigens and leprosy ([19, 41]), moreover, that HLA linked genes influence the clinical type of disease resulting from infection with *M. leprae* ([10]).

For these reasons we decided to study the occurrence of amyloidosis and some readily measurable serological parameters with possible relevance to amyloidosis in Australian Aboriginal patients with leprosy and their families. Because searches for a haplotype association and for HLA-D linkage were also objects of the study, we chose families in which there was more than one affected member. The transport of lymphocytes from the outback of the Northern Territory to Sydney at times impaired lymphocyte viability and made tissue typing difficult. Hence the genetic part of the study is still in progress and will be reported separately. However, the main part of the results are reported here. The picture which emerges is one of a population, clinically in good health and nutrition, but demonstrating a high frequency of subcutaneous amyloid deposits, humoral hyperreactivity in the form of non-organ specific autoantibodies and acute phase proteins, evidence of frequent exposure to hepatitis B virus (HBV) and treponemal infection, and almost universal exposure to hepatitis A virus.

MATERIALS AND METHODS

Subjects. In the family study, there were 70 persons belonging to 11 families. Since only families with more than one affected member were selected, this group contained 27 persons with leprosy of whom 11 were probands, 13 were first degree relatives, and 3 were spouses. The remaining 43 family members (35 first degree relatives, 3 second degree relatives, and 5 spouses) were unaffected. Specimens were collected during aerial visits to the mission stations of Roper River, Port Keats, and Oenpelli. They were processed the same day at the East Arm Hospital, Darwin. Lymphocytes were flown by commercial aircraft to Sydney and deep frozen sera to Adelaide, to Helsinki, Finland and Oslo, Norway. In addition, 26 patients with leprosy were included who were patients at the East Arm Hospital at the time of the study. They were not related to the subjects in the family study.

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TABLE 1. *Age and sex distribution and type of leprosy.*

Subjects	Total	Male	Female	Mean age ± S.D.	Type of leprosy		
					TT	BB	LL
All patients with leprosy	53	33	20	41.8 ± 13.3	20	18	15
EA patients[a]	26	18	8	42.0 ± 15.0	9	11	6
Family study	27	15	12	41.6 ± 13.8	11	7	9
Unaffected family members	43	18	25	23.4 ± 13.8	—	—	—

[a] Patients with leprosy hospitalized at the East Arm Hospital.

The type of leprosy was classified according to the clinical and histological criteria of Ridley and Jopling ([33]) so that the lepromatous group comprised patients with polar and borderline lepromatous disease, and the tuberculoid group those with polar and borderline tuberculoid leprosy (Table 1).

A control group of Aboriginal people was not available, and the reference ranges given in the tables are those of the participating laboratories. Where pertinent, statistical comparisons have been made between subjects with or without leprosy, with or without subcutaneous amyloid deposits, or between the two polar forms of leprosy.

Methods. Amyloid deposits were sought in fine needle aspirates from subcutaneous abdominal fat ([43]). Specimens stained with Congo red were examined, coded by the same observer on two separate occasions, and only those regarded as containing amyloid on both occasions were finally scored positive.

Immunofluorescent tests for antibodies to nuclear antigens (ANA), smooth muscle (SMA), mitochondria (AMA), liver-kidney microsomes (LKM), reticulin, gastric parietal cells (PCA), thyroid microsomes, epidermal intercellular junctions, and dermal-epidermal junction (skin basement membrane) were performed on acetone-fixed cryostat sections of rat liver and unfixed sections of rat liver and stomach, human thyroid and kidney and guinea pig lip. Sera were tested at an initial dilution of 1:10, and the same batch of polyvalent fluorescein-conjugated antihuman globulin (Wellcome Laboratories) was used throughout the study. Antibody to double stranded DNA (ds DNA) was detected by radioimmunoassay using ^{125}I labelled ds DNA (Amersham).

HB_sAg, the corresponding antibody (anti HB_s), and core antibody (anti HB_c) were sought by radioimmunoassay (Ausria II, Ausab and Corab, Abbott Laboratories). The e antigen (HB_eAg) was sought by counterelectrophoresis ([9]) and antibody to hepatitis A virus by radioimmunoassay (Hav-ab, Abbott Laboratories).

Antibodies to extractable nuclear antigens (ENA) were detected using double immunodiffusion ([40]). ENA, a mixture of saline soluble nuclear antigens, was extracted from acetone powder of rabbit thymus (Pel-Freeze Biologicals, Rogers, Arkansas), and sera which reacted with ENA were further characterized by counterelectrophoresis for the specificity of the antibodies for ribonucleoprotein (RNP), Sm antigen, and Ha antigen ([20]). Tests for antibodies to RNP and Sm were done before and after treatment of ENA with ribonuclease or heating (56°C, 150 min). Since RNP is RNase and heat sensitive ([1]), sera which reacted with untreated but not with RNase or heat treated ENA were regarded as containing antibodies to RNP. By contrast, Sm is both RNase and heat-resistant so that sera which precipitated with RNase and heat-treated ENA were regarded as positive for Sm antibodies. Purified Ha antigen (Teppo, A. M., *et al.*, unpublished data) was used to test sera for Ha antibodies.

Cryoglobulins. Cryoprecipitation of 0.5 ml samples was allowed to take place at 4°C for 5 days. Precipitates were separated by centrifugation at 5000 rpm for 10 minutes at 4°C and washed twice with 0.5 ml of 1 mM phosphate buffer, pH 6.2. They were dissolved in 0.1 ml of PBS at 37°C and assayed for immunoglobulins and Clq by double immunodiffusion against antihuman IgG, IgA, IgM (Orion Diagnostica, Helsinki), and antihuman Clq (Dakopatts, Copen-

hagen). Protein concentrations were determined by the Folin-Ciocalteau method.

α-Lipoprotein (α-LP) was assayed by electroimmunoassay as described by Ganrot ([13]) in agarose gel containing 3.5% antiserum (Behringwerke), and Clq was measured by radial immunodiffusion in 1% agarose gel in PBS containing 0.1 M EDTA and 0.4% antiserum (Dakopatts, Copenhagen). Standard serum for these two assays consisted of a pool of sera from 400 healthy blood donors (Finnish Red Cross, Helsinki) and reference values relate to 104 healthy persons. Serum amyloid associated (SAA) protein was measured by radial immunodiffusion in 1% agarose gel in 0.025 M barbital buffer, pH 8.6 ([15]), containing 15% antiserum to human AA produced in rabbits (courtesy Dr. Bjorn Skogen) according to the method of Anders, *et al.* (1977). Purified AA protein was used as the standard ([16]).

Serum C reactive protein (CRP) was measured by radial immunodiffusion in 1% agarose gel against 2% antiserum (Orion Diagnostica, Helsinki). Based on results in 104 healthy persons, values ≥10 mg/L were regarded as elevated.

Serum angiotensin converting enzyme activity (ACE) was measured spectrophotometrically ([21]) using hippuryl-L-histidyl-L-leucine as the substrate and serum lysozyme (LZM) was determined by the lysoplate technique using human LZM as the standard ([28]). The reference values for ACE are derived from 68 and those for LZM from 237 healthy Finnish persons.

RESULTS

Subcutaneous amyloid deposits. Aspiration was done in 91 persons, and satisfactory specimens of subcutaneous fat were obtained in 55. Amyloid was detected in 18 of these and, although it was more common in patients with leprosy than in unaffected family members, the association was not significant (Table 2). Moreover, there was no tendency for amyloid deposits to be associated predominantly with the lepromatous form of the disease or with the presence of long-standing trophic ulceration with or without osteitis.

Serum α-LP was significantly higher in patients than in unaffected persons (Table 3). However, although it was also higher in lepromatous than in tuberculoid leprosy and higher in subjects with subcutaneous amyloid than in those without, the differences did not reach statistical significance. Amyloid deposits and lepromatous leprosy were not associated with significantly raised SAA protein, and serum ACE and LZM were within normal limits with no significant differences between any of the groups studied.

TABLE 2. *Occurrence of subcutaneous amyloid deposits among the satisfactory samples.*

Subjects	Number of successful samples	Number of samples with amyloid detected
Unaffected family members	18	3
Patients with leprosy	37	15[a]
Lepromatous	12	3
Borderline	11	5
Tuberculoid	14	7

[a] $\chi^2_c = 2.14$, not significant, compared to unaffected family members.

Cryoproteins were found in trace amounts in nearly three-quarters of the sera but far less often in moderately large amounts. They were no more frequent in subjects with leprosy than in those without, nor in lepromatous than in tuberculoid leprosy. In all instances the cryoprotein contained IgG, but eight subjects had mixed IgG-IgA cryoproteins. IgM and Clq were not detected in any of the cryoprecipitates. Serum Clq was similar in patients with leprosy and healthy relatives, and there was no significant difference between tuberculoid and lepromatous leprosy. Serum CRP was elevated (≥10 mg/L) frequently but equally so in all groups studied.

Autoantibodies. SMA were more common than in the South Australian reference group and were detected with approximately equal frequency in patients with leprosy and unaffected family members (Table 4). Their presence was not linked with evidence of continuing HBV replication (data not shown). ANA were detected to moderately high titers in three subjects; all had subcutaneous amyloid, but none had anti ds DNA, nor was this antibody found in any person in the study. The only other immunofluorescent antibody which was found

TABLE 3. *Serum α lipoprotein (α-LP), serum amyloid associtated (SAA), lysozyme (LZM), angiotension-converting enzyme (ACE), cryoprotein, C1q, and C reactive protein (CRP).*[a]

Subjects	N	Serum α-LP (% normal serum)	SAA protein (mg/L)	LZM (μg/ml)	ACE (nmol/min/ml)	Cryoprotein (g/L) number of subjects			C1q (% normal serum)	CRP (mg/L)
						≥.1	≥.2	≥.3		
Unaffected family members	43	50.3 ± 15.2	15.9 ± 47.4	11.7 ± 3.1	26.8 ± 8.0	32	22	6	75%	14.4 ± 31.0
Patients with leprosy	50	61.8 ± 22.2[b]	4.8 ± 10.6	11.3 ± 3.1	23.6 ± 6.3	36	14	3	73%	7.3 ± 4.0
Lepromatous	15	61.9 ± 26.2	7.9 ± 18.1	11.2 ± 3.8	26.6 ± 6.2	11	4	2	84%	6.5 ± 2.0
Tuberculoid	18	53.6 ± 13.7	2.5 ± 3.1	10.9 ± 2.7	24.3 ± 7.9	13	3	1	68%	5.9 ± 2.0
Cutaneous amyloid										
Detected	15	63.1 ± 23.7	4.4 ± 10.5							
Not detected	34	53.8 ± 18.5	14.3 ± 39.8							
Reference range	104	115 ± 14	≤1[c]	8.9 ± 2.9	28.1 ± 6.0					

[a] Unless otherwise noted, values are given as mean ± S.D.
[b] Significantly different from unaffected family members, $p < 0.01$, Student's t test.
[c] Approximately 10% of healthy persons have values exceeding 1 mg/L (Natvig, personal communication).

in more than single subjects was the antibody reactive with the dermal-epidermal junction producing a tubular band of fluorescence similar to that seen in bullous pemphigoid. Satisfactory subcutaneous fat aspirates were obtained from only five of the ten subjects with the basement membrane antibody and contained amyloid in four.

Antibodies to ENA were present in eight of the 93 persons studied. Three sera contained antibodies against both RNP and Sm antigen, three against RNP alone, and one each against Sm antigen alone or Ha antigen alone. Five of the eight persons had leprosy and two subcutaneous amyloid. Three of the sera also contained ANA but only one to a high titer (1:1280). The remaining five sera also contained autoantibodies, four SMA, and one PCA.

TABLE 4. *Occurrence of autoantibodies (number of positive sera).*

Antibody	Patients with leprosy (N = 52)	Unaffected family members (N = 43)	South Australian blood donors (N = 206)
Smooth muscle	19	19	9
Antinuclear	4 (3[a])	1	7
Native DNA	0	0	0
Parietal cell	0	1	4
Thyroid	1	0	12
Mitochondrial	0	0	1
Reticulin	1	0	0
Skin basement membrane	5	5	5
Skin intercellular	1	0	1
Liver-kidney microsomal	1	1	0

[a] Titers 40, 320, 1280.

Communicable diseases. Evidence of past or present HBV infection was common (Table 5). Fourteen persons carried HB_sAg, and all but two of these had leprosy which was classified as tuberculoid in four, borderline in five, and lepromatous in three. This association was significant ($\chi^2_c = 4.978$, $p < 0.05$). The e antigen was detected in only one serum and anti e not at all. Anti HB_s and anti HB_c were found in approximately half of the subjects, the latter as an isolated finding without other HBV markers in 13 persons. Evidence of past infection with hepatitis A virus was almost universal and serological evidence of active syphilis was present in approximately half of the patients with leprosy.

DISCUSSION

The finding of subcutaneous amyloid deposits in as many as 15 of 37 patients with

TABLE 5. *Presence of serological markers of hepatitis B, hepatitis A, and treponemal infection (number of positive sera).*

Serological marker	Patients with leprosy (N = 52)	Unaffected family members (N = 43)	South Australian blood donors (N = 206)
HB_sAg	12	2	0
Anti-HB_s	26	23	5
Anti HB_{core}	29 (6)[a]	29 (7)[a]	2 (0)[a]
HB_eAg	0	1	0
Anti-HB_e	0	0	0
Anti-HA	49 (94%)	43 (100%)	91 (44%)
VDRL confirmed by FTA	25	5	0

[a] Figures in brackets denote number of sera with anti HB_{core} not accompanied by anti HB_s.

leprosy was unexpected as was the presence of amyloid at least as often in tuberculoid as in lepromatous leprosy. McAdam and Anders ([27]) found amyloid in rectal biopsy specimens from 16 of 90 patients, and 11 of the 16 had lepromatous disease with long-standing trophic ulcers as a possible cause for the amyloid in the remainder. On the other hand, Cathcart, *et al.* ([8]) found no correlation between the presence of amyloid and the type of leprosy in their group of 101 patients. They detected amyloid in gingival biopsy specimens from 17 patients, but since biopsies were not done in all patients, but only in those who had hepatosplenomegaly, albuminuria, or azotemia, the prevalence is likely to be an underestimate. Indeed, a further seven patients were thought likely to have amyloidosis on the basis of abnormal Congo red retention. In the present study, subcutaneous fat aspiration gave a disappointingly high rate of inadequate specimens—as noted by previous authors ([26]). Despite the exclusion of such samples and recording as positive only those which had been so scored on two separate occasions, the possibility remains that the method, through non-specific fluorescence of connective tissue fibrils, lacks the accuracy of rectal or gingival biopsy. These latter methods were, however, unacceptable to the Aboriginal population on cultural grounds. We do not know how often subcutaneous amyloid deposits occur in a random Aboriginal population, but their finding in 3 of 18 unaffected family members suggests that they are not rare. Indeed, McAdam ([26]), using subcutaneous fat aspiration, found a 7% prevalence of amyloid in a rural New Guinea population.

α-LP levels were low in the Aboriginal study population, but it was genetically and environmentally as dissimilar as possible from the Finnish Red Cross blood donors from whom the reference range was derived. Of greater interest in view of the recent report that amyloid protein AA precursor is one of the apoproteins of high density lipoproteins ([2]) is the significant increase in α-LP in patients with leprosy compared with unaffected family members. Although persons with subcutaneous amyloid also had higher α-LP levels than those without, the difference was not significant; this may relate to the small numbers studied. SAA protein did not distinguish between lepromatous and tuberculoid leprosy, unlike in a previous study ([34]), nor between the presence or absence of subcutaneous amyloid deposits. ACE and LZM were similarly unhelpful. Serum ACE is raised in sarcoidosis ([21,35,39]), but among other granulomatous disorders leprosy has been characterized by both elevated ([22]) and normal values ([39]). The present study showed no deviation of ACE from normal in any of the groups, nor were raised LZM values encountered as has been described in all forms of leprosy ([32]), although this claim has recently been disputed ([34]).

Tests for autoantibodies showed SMA in approximately 40% of patients and relatives. SMA have previously been described in 27%–64% ([11,31]) of patients with lepromatous leprosy and antibodies reactive with cytoskeletal intermediate filaments in 69% of patients with leprosy ([24]); these antibodies cross-react with smooth muscle ([23]). In the current study SMA were not associated with leprosy or with continuing HBV replication as evidenced by HB_sAg or by anti HB_e without anti HB_s and thus have to be ascribed to unidentified infective or inflammatory processes.

Organ-specific autoantibodies were very uncommon, but skin basement membrane antibodies were unexpectedly found in approximately 10% of patients and relatives.

They are generally considered to have a high degree of specificity for pemphigoid and to be restricted to the bullous diseases ([3]), although they have been described in 2 of 32 patients with lepromatous leprosy ([30]). There was no clinical evidence of pemphigoid or related diseases in any of the subjects of the present study, but we have recently encountered the antibodies in Aboriginals with other dermatological disease (Green and Wangel, unpublished observations).

Another unexpected finding, to our knowledge not previously reported in leprosy, was the presence of antibodies to ENA in eight sera. Of the antibodies to three of the well-characterized antigens of ENA, antibodies reactive with RNP are found most frequently in mixed connective tissue disease ([36]), those reactive with Sm antigen are most characteristic of systemic lupus erythematosus ([36, 40]), and antibodies against Ha antigen tend to be associated with Sjögren's syndrome ([1]). None of these conditions was obvious or known to exist in the persons of this study and no instance of anti ds DNA antibodies was found, suggesting that active SLE was not present in the study group. On the other hand, the presence of antibodies to one or more of the antigens of ENA was in all instances accompanied by other autoantibodies and was therefore part of a polyclonal response which, however, was not related to leprosy.

Measurements of serum Clq and analysis of cryoproteins did not prove rewarding. Cryoproteins, whether in trace or larger amounts, were frequent in the groups studied and, despite the care taken to store sera at −70° until analysis, the trace amounts may have been artifacts in the form of aggregated IgG rather than immune complexes. However, immune complexes do occur in sera from both polar forms of leprosy as shown by raised ^{125}I Clq binding in 82% of patients with lepromatous and 58% of patients with tuberculoid disease ([4]) and at least in lepromatous leprosy, are of the IgM-IgG type ([7]). The subjects of the present study had IgG or IgG-IgA cryoproteins and these were not related to the presence or absence of leprosy.

Exposure to HBV appears to have been equally common in patients with leprosy and in unaffected family members as judged by the presence of anti HB_s and anti HB_c. However, the HB_sAg carrier state was virtually confined to the patients. Impaired cell-mediated immunity and prolonged institutionalization are usually advanced as the reasons for an association of HB_sAg carriage and leprosy, particularly lepromatous leprosy, but the evidence for such an association is conflicting (reviewed by Sher, *et al.* ([38])).

Serological evidence of active syphilis was also more common in patients than in family members, but this was most likely due to the higher proportion of sexually mature persons in the former group.

The study thus demonstrated increased humoral reactivity, reflected by the presence of multiple autoantibodies, the acute phase proteins SAA and CRP, and cryoproteins in the Aboriginal population. However, both this increased reactivity and amyloid deposits were unrelated to any particular type of leprosy or even to leprosy itself, and are therefore presumably due to other factors. The nature of these factors was not made clear by the study, but chronic infection is one possibility. In the tropics, secondary amyloidosis may be due to leprosy, filariasis, and malaria, or it may occur without an obvious predisposing cause ([26]). Filariasis and malaria were not observed in this study but chronic sino-oto-pulmonary infections are common in Aboriginal communities and cause persistent immunological stimulation ([12, 17, 18]). Treponemal and HBV infection, so frequent in the present study, would be expected to reinforce this stimulation which, if prolonged, might provide the explanation for the unexpected finding of subcutaneous amyloid deposits in persons without leprosy.

SUMMARY

One group of 11 Aboriginal families, consisting of 27 persons with leprosy and 43 unaffected family members, and a second group of 26 patients with leprosy were studied in the Northern Territory of Australia. Amyloid deposits were sought in fine-needle aspirates of subcutaneous fat and serological investigations relevant to amyloidosis and to the humoral immune response were done. The study showed unexpectedly high frequencies of amyloid

deposits, evidence of persisting hepatitis B virus (HBV) infection, and antibodies to smooth muscle, to skin basement membrane, and to extractable nuclear antigens (ENA). Compared with unaffected family members, patients with leprosy had increased serum α-lipoprotein (α-LP) and were more often hepatitis B surface antigen (HB_sAg) carriers but, contrary to expectations, the presence of amyloid, the α-LP level, serum amyloid associated (SAA) protein, and the HB_sAg carrier state all appeared unrelated to the type of leprosy.

RESUMEN

Se estudiaron, un grupo de 11 familias aborígenes consistente de 27 personas con lepra y 43 familiares sanos, y un grupo adicional de 26 pacientes con lepra, en el territorio Norte de Australia. Se buscaron depósitos de amiloide en aspirados de grasa subcutánea y se hicieron investigaciones serológicas relevantes a la amiloidosis y a la respuesta inmune humoral. El estudio mostró elevadas frecuencias de depósitos de amiloide, evidencias de infección persistente por el virus de la hepatitis B (HBV), presencia de anticuerpos contra músculo liso, contra membrana basal dérmica, y contra antígenos nucleares extractables (ENA). Comparando con los familiares no afectados, los pacientes con lepra tuvieron aumentados los niveles de α-lipoproteína sérica (α-LP) y fueron portadores más frecuentes del antígeno superficial del virus de la hepatitis B (HB_sAg) pero, contrario a lo que pudiera esperarse, la presencia de amiloide, el nivel de α-LP, el nivel de proteína sérica asociada al amiloide (SAA), y el estado de portador de HB_sAg, no parecieron estar relacionados con el tipo de lepra.

RÉSUMÉ

Dans le Territoire du Nord (Northern Territory) en Australie, on a étudié un groupe de 11 familles d'autochtones, comprenant 27 personnes atteintes de lèpre et 43 personnes indemnes, et un groupe témoin de 26 malades atteint de lèpre. Des dépôts amyloïdes ont été recherchés au moyen d'aspirations pratiquées avec une aiguille fine dans la graisse sous-cutanée; on a également procédé à des investigations sérologiques en rapport avec l'amyloïdose et la réponse immunitaire humorale. De manière inattendue, cette étude a révélé des fréquences élevées de dépôts amyloïdes, des signes d'une infection persistente par le virus de l'hépatite B (HBV), et des anticorps dirigés contre les muscles lisses, la membrane basale cutanée, et les antigènes nucléaires extractables (ENA). Lorsqu' on les compare avec les membres familiaux indemnes de lèpre, les malades souffrant de lèpre présentaient une augmentation des α-lipo-protéines (α-LP) du sérum et étaient plus fréquemment porteurs de l'antigène de surface pour l'hépatite B (HB_sAg); par ailleurs, et contrairement à ce que l'on aurait pu attendre, la présence de dépôts amyloïdes, les taux d'α-LP, la protéine associée à l'amyloïde du sérum (SAA), et la fréquence de portage pour HB_sAg se sont révélés sans relation avec le type de lèpre.

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