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Circulating and Tissue Immune Complexes in Leprosy[1]

Shobha Sehgal and Bhushan Kumar[2]

Alterations in the immunological reactivity of the host due to infection by leprosy bacilli have been intensively investigated by a number of authors ([21, 22]). The major emphasis, however, has been on the thymic dependent lymphocytes as evidenced by diminished blast transformation to specific and nonspecific antigens, depressed lymphokine production, and diminished *in vivo* skin sensitization ([2, 4]). On the other hand, the leprosy patient's capacity to produce immunoglobulins is supernormal ([3, 4, 8, 18]). This state of selective depression of cell-mediated immunity (CMI) and elevation of humoral antibodies is akin to that observed in some autoallergic diseases. Further, the incidence of various autoantibodies is markedly enhanced in lepromatous leprosy ([1]). Complement levels are reported to be normal in all forms of this disease except raised C_2 and C_3 in erythema nodosum leprosum (ENL) ([17, 24]). Immunofluorescence studies using biopsy material from patients with ENL have revealed the presence of immunoglobulins and complement; however, detailed studies pointing to a direct Type III hypersensitivity mechanism in ENL are lacking. Quismorio, *et al.* ([11]) reported conspicuous IgM staining at the dermoepidermal junction in apparently uninvolved skin in the majority of lepromatous patients. In the present study, skin biopsies have been studied for the presence of such tissue immune complexes, and the sera of patients have been screened for various autoantibodies and for the presence of circulating immune complexes.

MATERIALS AND METHODS

Patients. Serum levels of IgG, IgA, and IgM were estimated in 40 leprosy patients living in a Haryana leprosarium during the year 1970 (Group A). The method has been described earlier ([15]). All patients studied had active lepromatous leprosy (LL) ([12]) and Bacteriologic Indexes between 2+ and 5+. All the patients had been on sulfone therapy for a variable period, and all were adequately treated for intestinal parasites, if any.

The rest of the investigations were done on the sera of 26 patients attending the dermatology clinic of the Nehru Hospital during 1978–1979 and will henceforth be referred to as Group B. Of the 26 cases four had BL disease on the Ridley-Jopling scale ([12]), and the rest were all of the LL type. Six of the 26 patients had ENL. Most of them had received short or long term sulfone therapy. IgD estimations were done using radial immunodiffusion. Antisera were obtained from the WHO Reference Centre, Lausanne. Results were compared with normal controls from the laboratory.

Tissue antibodies. A composite block including mouse stomach, liver, kidney, and human thyroid was embedded in Ames OCT compound, and 5 μ thick frozen sections were cut in a cryostat. The sections were layered with a 1:10 dilution of patient's serum for 30 min at 37°C, washed in several changes of phosphate buffered saline (PBS), and treated with an appropriate dilution of fluorescein labeled, anti-human immunoglobulins (heavy chain specific, Hyland Division, Travenol Laboratories, U.S.A.) for another 30 min. The slides were mounted in buffered glycerine after several washings and viewed with a Reichart microscope using E3 exciter and SP3 barrier filters. The intensity of the fluorescence was arbitrarily graded as 1+ to 4+. Thyroglobulin and thyroid microsomal antibodies were estimated by an indirect hemagglutination reaction using a kit provided by Fujizoki Pharmaceuticals, Japan. The last dilution giving a positive reaction was expressed as the titer.

Sera were scanned for rheumatoid arthritis (RA) activity using Rheuma latex (Burroughs Wellcome, Research Triangle Park,

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[2] S. Sehgal, M.D.; B. Kumar, M.D., Department of Immunopathology and Dermatology, Postgraduate Institute of Medical Education and Research, Chandigarh-160 012, India.

North Carolina, U.S.A.) and for the presence of HB_SAg by counterelectrophoresis; antiserum was obtained from the Australian Red Cross Centre.

Rubino test. Twenty selected samples were also subjected to the Rubino test according to the method described by Wager, *et al.* ([23]). Briefly, human group O Rh+ red cells were washed in saline and a 5–10% formaldehyde solution (35%) allowed to interact with them for 24–48 hr until the cells turned brown. These were washed thoroughly 5 times in saline and made into a 50% suspension in PBS (pH 7.2) and stored at 4°C. For the test proper, 25 μl of a 2.5% cell suspension was mixed with an equal volume of doubling dilutions of patients' sera in microtiter plates and sedimentation patterns read after 4 hr of incubation at room temperature. Spreading was taken as positive while button formation was taken as negative. The last dilution giving spreading was taken as the titer.

Direct fluorescence. This test was employed to study skin immune complexes. Frozen sections of skin biopsies taken from the edge of clinically affected areas were treated with fluorescein labeled antibodies against human IgG, IgA, IgM, and β_1c. Details of the method have been published elsewhere ([16]).

Platelet aggregation test (PAT). The platelet aggregation test was performed according to the method advocated by Pentinnen ([10]). Briefly, 20 ml of heparinized blood was drawn in a plastic syringe and transferred to a disposable centrifuge tube. Platelet rich plasma was collected after immediate centrifugation. Platelets were washed twice with pyrogen free saline and once with buffered salt solution (Ca^{++} and Mg^{++} free with 1.0 mM glucose at pH 7.8) and suspended in PBS at a final concentration of 200,000/mm^3. The test was performed in "U" microplates when 0.25 ml of platelets were added to an equal volume of doubling dilutions of patient's serum. The sedimentation pattern was recorded after incubating the plates for 18 hr at 4°C; the buttons were read as negative.

Anticomplementary activity (complement deviation test). Washed sheep red cells were sensitized with an optimum dilution of amboceptor and 120 million (EA) cells labeled with 100 μCi of ^{51}Cr (sodium chromate) according to the method of Sanderson, *et al.* ([14]). The washed, sensitized, labeled erythrocytes were adjusted to a final concentration of 120 million/ml. In small round bottom tubes the reagents were then added in the following order:

1. 50 μl heat inactivated serum + 50 μl CFT buffer[3]	30 min at 4°C
2. + 100 μl complement[4]	15 min at 4°C
3. + EA[5] 2.5 million + 250 μl CFT buffer[3]	15 min at 37°C

The tubes were then centrifuged and the radioactivity in the supernatants determined in a gamma counter (Model 4601, Nuclear Enterprises, Edinburgh, U.K.). Controls consisted of EA incubated in distilled water for maximal release and in CFT buffer for spontaneous release. The percentage inhibition of chromium release was then calculated after appropriate corrections.

All samples were coded and the results of the various investigations subjected to statistical analysis using the Student's *t* test.

RESULTS

Group A. The immunoglobulin levels revealed a polyclonal rise. The mean (±S.D.) for IgG was 2128 (±556) mg%, IgA 519 (±83) mg%, and IgM 209 (±82) mg%. The control values are given in Table 1. The differences were highly significant for all three immunoglobulin classes. Serum IgD levels for patients in Group B did not reveal any significant difference from the controls. The mean value in the control group was 27.60 (±19.57) mg% as compared to 30.45 (±20.40) mg% in the patients.

Nine out of 26 patients showed a positive Rheuma Latex reaction (dilution > 1:20); the difference was statistically higher than in the control group. The titer was more than 1:640 in three patients. Smooth muscle antibodies could be detected in five out of 26 patients, but the reactions were weak, the titers varying between 1:20 and 1:60.

[3] CFT buffer = Complement Fixation Test buffer (0.1 M barbital buffer, pH 7.2).

[4] 1:30 dilution of fresh guinea pig serum was used as a source of complement.

[5] EA = amboceptor treated erythrocytes.

TABLE 1. *Serum immunoglobulin concentrations. Values are mean ± S.D. in mg percent. IgG, IgA, and IgM concentrations were determined in Group A patients and controls. IgD concentrations were determined in Group B patients and controls.*

Individuals	IgG	IgA	IgM	IgD
Lepromatous leprosy	2128 ± 556[a] N = 40	519 ± 83[a] N = 40	209 ± 82[a] N = 40	30.40 ± 20.40 N = 26
Normal controls	1145 ± 330 N = 118	273 ± 119 N = 118	144 ± 60 N = 118	27.60 ± 19.57 N = 30

[a] $p < 0.001$, Student's t test, compared to controls.

One patient showed a titer greater than 1:80 and was in the transition phase of BL-LL. Parietal cell antibodies were present in 10 out of 26 patients. In the control group the frequency is up to 15% in the local population. Thus, the frequency of parietal cell antibodies was higher in patients than in the control group. Surprisingly, antinuclear factor (ANF) was positive in only one patient. In this patient we could also demonstrate "*in vivo*" antinuclear antibody as judged by the direct immunofluorescence on the skin biopsy (Fig. 1). Similarly, the frequency of HB_sAg was low. Only case no. 9 showed a positive reaction with counterelectrophoresis. Radioimmunoassay could not be done. The frequency in the control population is 2.3% (Pal, S. R., personal communication). Levels of C_3 were decreased in 10 out of 25 patients tested when less than 60% of the mean of the normals was taken as the cutoff point. No patient showed an increased level (Fig. 2). The two patients showing the lowest levels of C_3 were not having ENL reaction at that time. Renal biopsies were examined in three autopsy cases, but the glomeruli failed to reveal immune complexes. However, arteriolar staining for IgG and C_3 was seen in one case (Fig. 3).

Skin immunofluorescence. A total of 17 cases and 20 tissues were tested. Three were repeat samples. Positive "*in vivo*" antinuclear antibody was observed in case no. 9. Incidentally, he also showed staining of IgM at the dermoepidermal junction (DED). In all other cases DED staining was negative. Complement could be demonstrated in 6 of 20 specimens in the dermal vessels. Two of the earlier biopsies of case no. 16 were negative. However, she became positive when she developed ENL. IgG could be detected in 6 of 17 cases. In one case, the staining pattern was also observed in the intercellular substance. Thus complement, IgG, and IgM were detected with equal frequency (35%), but the incidence of immune complexes at the DED was very low. Three cases with ENL were negative. The most common sites of immune complexes were dermal capillaries and arterioles, not large vessels (Table 2).

Demonstration of circulating immune complexes. As measured by the platelet aggregation test (PAT) and complement deviation tests, there was a very high incidence of circulating immune complexes. More than 50% inhibition of hemolysis was observed in the sera of 17 out of 20 cases (85%) tested for anticomplementary activity (Fig. 2). The platelet aggregation test showed a titer of less than 8 in all normal controls while in patients the titers varied between 4 and 256. The mean titer was 31 in patients compared to 1.2 in normal controls. The titers were, however, much lower than those observed in autoimmune diseases or kala-azar (Table 3). There was no direct correlation between the PAT titers and anticomplementary activities.

Rubino test. This test was performed in sera from 21 patients. The titers ranged from negative to more than 256 with a mean titer of 132. Fourteen of 21 patients showed a titer of 16 or greater. In the control group the mean titer was only 2.6. Interestingly, positivity was not restricted to lepromatous leprosy but was also noticed in some patients with chronic renal failure awaiting transplant surgery when 3 of 20 patients had titers greater than 16.

DISCUSSION

From the data presented it appears that there is a variety of humoral aberrations in lepromatous leprosy. The polyclonal rise of all the three immunoglobulins is in accor-

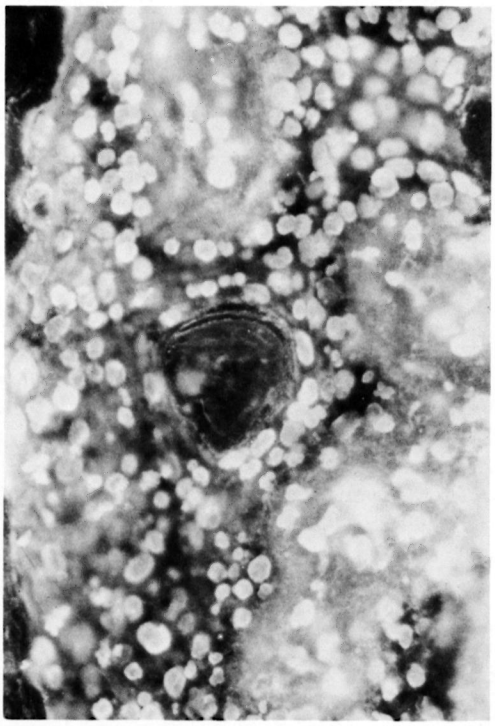

FIG. 1. Skin biopsy treated with fluorescein labeled anti-IgG showing Antinuclear Antibody reaction "*in vivo*" (direct fluorescence in skin biopsies). (×580).

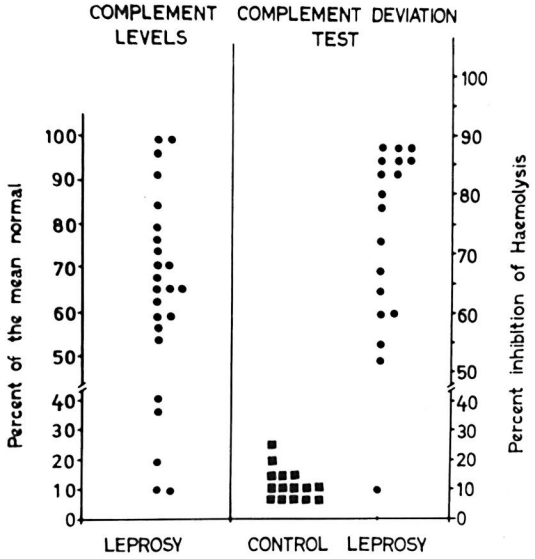

FIG. 2. C_3 levels and complement deviation tests in leprosy patients.

dance with the observations of Bullock, *et al.* [3]. However, in the studies reported by Sheagren, *et al.* [18], the IgM levels were not significantly raised. Lim and Fusaro [6] reported raised IgG and IgM but normal IgA levels. In our earlier studies we have documented higher IgM levels in normal Indians as compared to Caucasians [15]. It is quite likely that there were either occult stimuli or genetic factors responsible for the raised IgM in these patients. Thus, the levels would differ according to type of patients on the Ridley-Jopling scale included in this study, ethnic variations, and the therapy instituted. The levels of IgD were not statistically different from those of the control group.

The results of autoantibodies indicate that the incidence of parietal cell antibodies is significantly increased in lepromatous leprosy. Such observations have been recorded earlier by us [27] and other workers, including Wager, *et al.* [23] and Masala, *et al.* [7]. Studies of gastric functions in leprosy have not revealed any significant alterations (Kumar, B., unpublished observations). However, it is quite possible that iron deficiency, which is so common in India, may lead to mild inflammatory lesions of the mucosa and the release of parietal cell antigens without overt functional aberrations.

Although the frequency of positive latex agglutination reactions is higher in these patients, the frequency of antinuclear antibodies or the frequency of full blown systemic lupus erythematosus or rheumatoid arthritis is not increased. The situation is akin to malaria, where autoantibodies are frequently encountered, yet a full-fledged autoimmune disease association is not documented either in animals or man [26]. Similarly, thyroglobulin antibodies as studied by gel diffusion and microhemagglutination techniques were all negative. Thyroid microsomal antibodies were detected in 3 of 20 patients. The findings are in accordance with our earlier observations where a detailed hormonal assay was also conducted including T_3 and T_4 levels and no abnormality could be detected [6].

The much talked about IgG staining at the dermoepidermal junction was persistently negative in our patients, except one case, irrespective of whether the biopsy was from a clinically involved area or an uninvolved area of the skin. Quismorio, *et al.* [11] reported positive reactions in more than

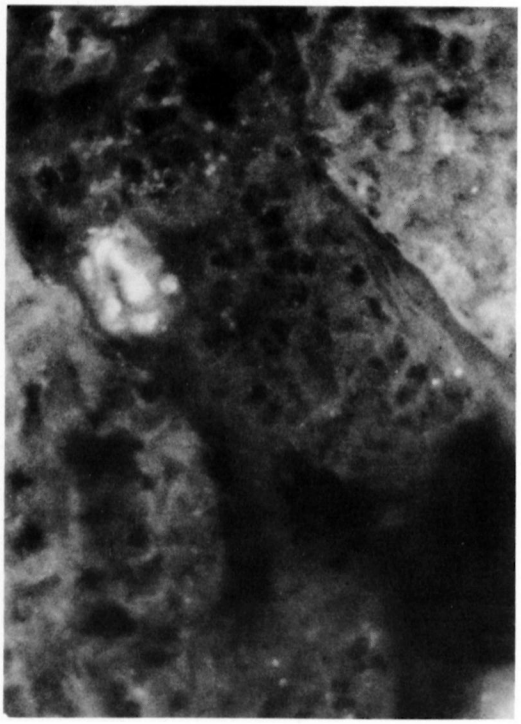

FIG. 3. Direct immunofluorescence of kidney tissue of an LL patient showing complement deposition in the arteriole. The glomeruli are free of any deposits (×580).

80% of leprosy patients. The reason for such a difference is not clear. It must, however, be stressed that in our hands the frequency of a positive lupus band test in Indians is also low when ANF titers are usually very high. It is quite likely that prolonged exposure to ultraviolet light may influence staining at the dermoepidermal junction, or excess melanin may influence the deposition at that site. The finding of *in vivo* ANF is interesting. Such a phenomenon could either be due to entry of preformed immune complexes into viable cells or due to local binding of antibody to the altered nuclear antigens of a damaged skin. Earlier, Wells, *et al.* [25] have also shown positive ANF "*in vivo*."

The immunofluorescence reaction in vascular lesions of the dermis was much less intense in ENL than the lesions of, for example, subcutaneous nodules of rheumatic activity. The complexes were more often encountered in small or medium sized vessels than in larger vessels. Four cases of frank ENL were clearly negative. Histologically, the lesions showed a predominant polymorphonuclear leukocytic reaction rather than fibrinoid degeneration. It may be that small amounts of complexes are rapidly cleared by a massive polymorphonuclear leukocytic reaction.

The presence of circulating immune complexes in leprosy has earlier been documented by Moran, *et al.* [9], Lambert, *et al.* [5] and Wager, *et al.* [23]. In a collaborative study Lambert, *et al.* [5] highlighted the utility of the platelet aggregation test in distinguishing LL from TT and claimed that this test was superior to the Clq deviation assay [19], Raji cell assay [20], and Clq binding assay [28]. Wager, *et al.* [23] reported a positivity of 53% in all the lepromatous spectra which was distinctly higher than in the tuberculoid spectra (3%, 14%). In the normal Brazilian controls the positivity rate was 9%, and in Finnish controls it was 1%. They also noticed that prior treatment of serum with Mitsuda lepromin interfered with the positivity.

Another interesting fact has emerged about the Rubino test, namely that it is not unique to lepromatous leprosy, at least not in this geographic region. We have found a positive Rubino test in patients with renal failure and in a patient with kala-azar. This time honored test is still mysterious but is regarded as being fairly specific for lepromatous leprosy. In chronic renal failure and lepromatous leprosy there is depression of T-cell function which also includes suppression of suppressor cells to some extent. It is quite likely that aberrations of suppressor cells may lead to the formation of agglutinating antibodies to the modified group O Rh positive cells which are used for the Rubino test. Since the basis for this test is not clear, further attempts are being made to explore this particular reaction.

SUMMARY

Various immunological studies were conducted in lepromatous leprosy patients. There was a polyclonal rise of all the three major immunoglobulins. Serum IgD levels remained unaltered. Positive latex agglutination reactions (RA) and parietal cell antibodies were distinctly more frequent in patients. Eighty-five percent of patients

TABLE 2. *Immunofluorescent studies of skin biopsies from Group B BL and LL patients.*

Case no.	ENL	Staining with antibodies against human				Location
		IgG	IgA	IgM	β_1c globulin	
1	−	+	−	+	+	[a]
2	−	−	−	−	−	−
3	−	−	−	−	−	−
4	+	−	−	−	−	−
5	−	−	−	−	−	−
6	−	−	−	−	−	−
7	+	−	−	−	−	−
8	+	−	−	−	−	−
9	−	+	+	+	+	[b]
10	−	−	+	+	−	[a]
11	−	−	−	−	+	[a]
12	−	+	−	+	−	[c]
13	−	−	−	−	−	−
14	+	+	−	−	+	[a]
15	+	+	−	+	+	[a]
16	+	+	−	+	+	[d]
17	−	−	−	−	−	−
Total positive/17	6	6	2	6	6	

[a] Blood vessels.
[b] Antinuclear antibody was demonstrable "*in vivo*," immunofluorescence localized to blood vessels and dermoepidermal junction.
[c] Blood vessels and dermis.
[d] Blood vessels and epidermis.

showed a strongly positive complement deviation test, and the mean platelet aggregation titer was 1 in 81 in the patients compared to 1 in 3 in the controls, thus indicating the presence of circulating immune complexes in a large percentage of cases.

Immunoglobulins and complement were

TABLE 3. *Platelet aggregation test titers in lepromatous leprosy patients' sera compared with other disease states and healthy controls.*

Patients (N)	Geometric mean titers (range)	Arithmetic mean titers (±S.D.)	% above maximum titers of healthy controls (8)
Kala-azar (15)	61.2 (0–512)	191 (216)[a]	73
Chronic active hepatitis (11)	60.1 (4–512)	209 (242)[b]	64
Chronic renal failure (22)	49.3 (4–512)	125 (168)[a]	73
Systemic lupus erythematosus (18)	40.3 (4–512)	148 (163)[a]	67
Lepromatous leprosy (20)	12.6 (4–256)	31 (60)[c]	45
Healthy controls (20)	0.5 (0–8)	1.2 (2.7)	—

[a] $p < 0.01$, Student's t test, compared to healthy controls.
[b] $p < 0.02$, Student's t test, compared to healthy controls.
[c] $p < 0.05$, Student's t test, compared to healthy controls.

observed in the dermal capillaries and small arterioles, but not in larger vessels, in 6 of 17 cases. Only one case showed staining of the dermoepidermal junction. Antinuclear antibodies (ANA) were detected in a single patient, and this patient also showed ANA *in vivo*.

RESUMEN

Se efectuaron varios estudios inmunológicos en pacientes con lepra lepromatosa. Se encontró una elevación policlonal de las 3 inmunoglobulinas mayores (IgG, IgM, IgA) en tanto que los niveles séricos de IgD estuvieron normales. En los pacientes, las pruebas positivas para la aglutinación del latex (AR) y para anticuerpos anti-células parietales, fueron claramente más frecuentes. Ochenta y cinco porciento de los pacientes dieron una prueba de desviación del complemento fuertemente positiva, y el título promedio de la agregación plaquetaria fue de 1 en 81 en los pacientes y de 1 en 3 en los controles; ésto indíca la presencia de complejos inmunes circulantes en un alto porcentaje de los casos. En 6 de 17 casos se observaron inmunoglobulinas y complemento en los capilares y en pequeñas arteriolas dérmicas pero no en los vasos más grandes. Sólo un caso mostró tinción de la unión dermoepitelial. En un sólo paciente se detectaron anticuerpos antinucleares (ANA) y este paciente también tuvo ANA *in vivo*.

RÉSUMÉ

On a mené une série d'études immunologiques chez des malades atteints de lèpre lépromateuse. On a observé une augmentation polyclonale de toutes les trois immunoglobulines principales. Les taux sériques d'IgD sont restés inchangés. Des réactions positives pour l'agglutination au latex (RA) et la présence d'anticorps cellulaires pariétaux, étaient nettement plus fréquentes chez les malades. Chez quatre-vingt cinq pour cent des malades, on a observé une épreuve de déviation du complément fortement positive, et le titre moyen d'agrégation des plaquettes était de 1 sur 81 chez les malades, comparé à 1 sur 3 chez les témoins. Ceci indique, dans une large proportion des cas, la présence de complexes immuns circulants. Chez six des 17 cas, on a observé des immunoglobines et du complément dans les capillaires du derme et dans les petites artérioles, mais non dans les vaisseaux plus larges. Il n'a été possible de colorer la jonction dermo-épidermique que dans un seul cas. Des anticorps antinucléaires (ANA) ont été détectés chez un malade seulement, et ce malade présentait également ANA *in vivo*.

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