Antinuclear Antibody and Rheumatoid Factor Estimations in the Diagnosis of Rheumatic Diseases in Northern India*

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SUMMARY **One hundred serum samples from North Indian patients with systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) and 51 samples from patients with other systemic connective tissue diseases (OCTD) such as systemic sclerosis, overlap syndrome and dermatomyositis were studied for the prevalence and titre of antinuclear antibody and rheumatoid factor expressed in International units. The results were compared with those obtained in 50 patients with non-rheumatic diseases (disease controls) and 120 healthy controls. None of the controls, diseased or healthy, showed an ANA titre of 15 SEAPAL units (1: 20) or greater. With increasing ANA titre beyond this level, there was a greater possibility of the disease being SLE or OCTD but not RA. However, the titre of ANA *per se* could not discriminate between SLE and OCTD. Similarly for RF, a titre of 30 IU/ml or more could be used to discriminate between connective tissue disorders and other diseases; the diagnosis of rheumatoid arthritis was most likely at a titre of RF above 60 IU/ml.**

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Auto-immune serum markers, namely antinuclear antibody (ANA) and rheumatoid factor (RF), are useful laboratory parameters in the diagnosis, differential diagnosis and follow-up of patients with systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and other systemic connective tissue diseases (OCTD).[1-5] However, it has been realised that these markers may also be detected in varying amounts in normal healthy controls as well as in people with other unrelated diseases.[6] Moreover, there is a possibility of ethnic and geographical variations in these parameters.[7] Therefore, it is essential to investigate the distribution of ANA and RF in normal healthy individuals of a certain ethnic group in a given geographic area and also to find out the extent of the parameters in rheumatic-immunological and non-rheumatic-nonimmunological diseases in the same ethnic group in a given geographic area. Such studies are essential for finding out the normal levels, relevant cut-off points and the sensitivity and specificity of these tests in relation to the rheumatic-immunological diseases.

In the present study, an attempt has been made to standardise these tests, using available international reference standards, in Northern Indian population which is more or less a homogeneous ethnic group of brown Caucasians.

PATIENTS

The subjects studied included the following groups:

(a) 120 normal healthy controls: There were 68 males and 52 females; their ages ranged from 3 to 75 years for males and from 6 to 70 years for females; the median age was 30 years. Of the subjects, 9 per cent were in their first decade, 6 per cent in their second decade, 58 per cent in their third decade, 14 per cent in their fourth decade, 2 per cent in their fifth decade, 5 per cent in their sixth and seventh decades and 1 per cent in the eighth

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decade. These subjects included 15 staff members working in various areas of the All-India Institute of Medical Sciences, 50 healthy blood donors, 30 subjects undergoing a routine serological test for syphilis (VDRL testing), 13 healthy elderly individuals undergoing cataract surgery and 12 healthy children having a routine medical check-up and receiving vaccinations.

(b) 50 disease controls: There were 19 males ranging in age from 4 to 79 years and 31 females ranging in age from 19 to 65 years; the median age was 40 years. The distribution of these subjects in successive decades from the first to the eighth decade was 8%, 8%, 20%, 10%, 18%, 24%, 10% and 2% respectively. The subjects were patients with a variety of common non-rheumatic and non-immunological diseases commonly seen in a big general hospital; the diseases included tuberculosis, essential hypertension, coronary artery disease, pyelonephritis, non-Hodgkin's lymphoma etc.

(c) 100 patients with systemic lupus erythematosus (SLE): The diagnosis of 92 females and eight males ranging in age from 8 to 60 years was made according to the 1971 American Rheumatism Association classification criteria from SLE.[8] The median age was 24 years and the majority of patients were in their second and third decades.

(d) 100 patients with rheumatoid arthritis (RA): The diagnosis of 16 males and 84 females ranging in age from 19 to 70 years was as per the 1958 revised ARA criteria with the majority in the category of "definite" RA after careful exclusion of non-RA inflammatory polyarthritides.[9] The median age was 40 years and the majority of these patients were in their third, fourth and fifth decades.

(e) 51 patients with other systemic connective tissue diseases (OCTD): including progressive systemic sclerosis (30), diagnosed as per ARA criteria,[10] undifferentiated connective tissue disease (19) and Sjogren's syndrome (2), diagnosed as per standard text-book criteria.[11] There were 47 females and four males, ranging in age from 15 to 55 years. The median age was 35 years and the majority were in their third and fourth decades. Serum samples were collected from patients after they were diagnosed to have SLE, RA or OCTD. None of the patients was on or had been on steroid therapy when the samples were drawn.

METHODS

Indirect Immunofluorescence Test for ANA

Standard indirect immunofluorescent test was used throughout the study. Unfixed cryostat sections of rat liver and kidney were used as substrate antigens. Serum dilutions were layered on these tissue sections on microscopic slides, inclubated for 30 minutes in a humidified chamber and washed three times in phosphate buffered saline (pH 7.40, 0.1 M); kept in motion by a mechanical stirring device. Polyspecific antihuman immunoglobulins (IgG, IgA, IgM) antiserum raised in goats and conjugated with fluorescein isothyocyanate (FITC) was obtained commercially (M/s Immunodiagnostics, Delhi, India). Optimal dilution was determined by "chequerboard" titration with a known antinuclear positive serum.[12] The highest dilution of FITC-conjugate giving a clear, positive test was considered "optimal" and used in the test. The serum-treated and -washed tissue sections were then layered with optimally diluted FITC conjugate and incubated for 30 minutes in a humidified chamber and washed as above. After the final wash, the slides were mounted in glycerol saline buffer and read under epifluorescent light using a Nikon 'Optophot' microscope.

A set of four reference standard sera containing 2.5 units/ml, 7.5 units/ml, 15 units/ml and 30 units/ml respectively (kindly provided by Prof. R.L. Dawkins, Department of Clinical Immunology, Royal Perth Hospital, Perth, Australia), referred to as SEAPAL units in this paper, were initially prepared in different dilutions to plot the standard curve. The reciprocal of the highest dilution of the test sera as converted into units and, when necessary, the standard curve was extrapolated. The highest dilutions in which the standards of 7.5 units/ml, 15 units/ml and 30 units/ml were positive, were 1:10, 1:20 and 1:40 respectively; the standard of 2.5 units/ml was negative even when applied undiluted. All control and test sera were screened at an initial dilution of 1:10. If a serum was negative at 1:10, the test was repeated (neat). If ANA was positive in neat serum only, the result was expressed as < 7.5 SEAPAL units/ml.

Latex fixation test for RF

Latex-RF kits (some purchased from M/s Wellcome Reagents, U.K., and others generously provided by M/s Hoechst Pharmaceuticals Ltd., Behring Diagnostics, India) were used throughout this study. A known reference standard provided by the manufacturers, as well as a WHO reference laboratory standard for RF (generously provided by WHO International Laboratory for Biological Standards, Statens Serum Institute, Copenhagen, Denmark) were simultaneously run with each batch of tests and the results expressed in international units (IU)/ml.

RESULTS

ANA was not detected in any normal control at the minimum detection level of 7.5 SEAPAL units/ml (1:10 dilution) or less. In the disease control group, five (10%) of the subjects showed a positive ANA test; two of them were positive at a titre of less than 7.5 SEAPAL units/ml. One of the latter subjects was a 24-year-old male with untreated malignant hypertension and the second was a 51-year-old

male who had undergone a coronary bypass procedure. Three patients, a 58-year-old male with non-Hodgkin's lymphoma, a 53-year-old male with renal calculus and a 56-year-old female with generalized tuberculosis, were positive at a titre of 7.5 SEAPAL units/ml. The highest incidences of ANA were seen in SLE and OCTD (Table 1).

The distribution of ANA titres in subjects in different categories, is expressed in a composite way graphically in Figure 1 using cumulative frequencies. A titre of 7.5 SEAPAL units/ml or more excluded all normal subjects and 94 per cent of the disease controls but yet included 99 per cent of the SLE and 98 per cent of the OCTD patients. Therefore, this was considered as the optimal starting screening titre of the serum samples which would exclude the 'background noise" and yet be highly sensitive for the diagnosis of SLE.

Similarly, a titre of 75 SEAPAL units/ml (1:100 dilution) or more was found to exclude up to 90 per cent of the RA patients and yet detected 84 per cent of the patients with SLE and 65 per cent of those with OCTD.

Table 2 gives the results of RF. The highest incidence and titres were seen in RA. The distribution of RF titre in subjects in different categories is expressed in a composite way graphically in Figure 2 as in Figure 1. A titre of 30 or more IU/ml excluded 100 per cent of the normal subjects, 97 per cent of the SLE patients, 78 per cent of those with OCTD and 98 per cent of those with non-rheumatic nonimmunological diseases and yet it detected 69 per cent of the patients with RA. At a titre of 60 or more IU/ml, 53 per cent of the RA patients were positive but almost all other groups were excluded. Thus, a titre of 30 IU/ml RF was considered sufficiently sensitive for the detection of RA and other related connective tissue diseases, and a titre of 60 or more IU/ml was considered highly specific for seropositive RA.

Table 1 Antinuclear antibody in healthy and diseased Indians.

Titres (SEAPAL unit/ml)	Normal subjects (n=120)	RA (n=100)	SLE (n=100)	OCTD (n=51)	Unrelated diseases (n=50)
<7.5	100*	65	1	2	94
7.5	0	20	5	25	6
15	0	0	2	0	0
37.5	0	5	8	8	0
75	0	3	17	14	0
150	0	4	13	15	0
225	0	0	10	6	0
300	0	2	15	12	0
375	0	1	15	6	0
600	0	0	9	4	0
750	0	0	3	8	0
900	0	0	1	0	0
1,125	0	0	1	0	0

*Percentage

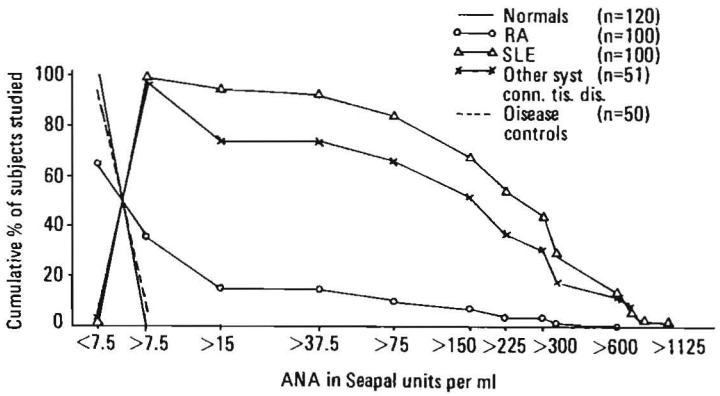

Fig. 1 Antinuclear antibody

Table 2 Rheumatoid factor in healthy and diseased Indians.

Titres (International units/ml)	Normal subjects (n=120)	RA (n=100)	SLE (n=100)	OCTD (n=37)	Unrelated diseases (n=50)
<15	98*	10	88	70	92
15	2	21	9	8	6
30	0	16	2	5	0
60	0	18	1	5	0
120	0	20	0	11	2
240	0	9	0	0	0
480	0	6	0	0	0

*Percentage

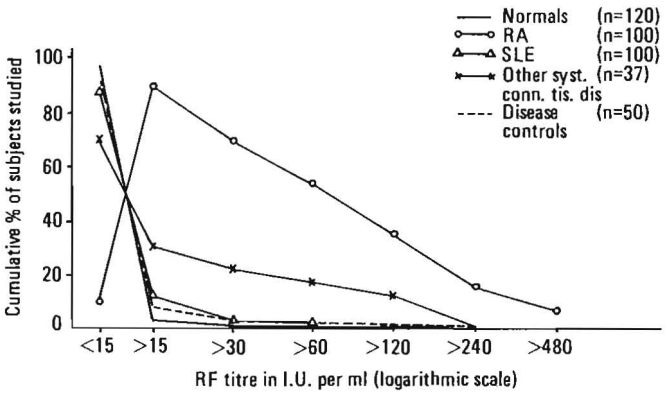

Fig. 2 Rheumatoid factor

DISCUSSION

It has been well brought out in the past that medical laboratory immunology, as is the case with any type of laboratory medicine, would become useful only if the specificity and sensitivity of the test being employed are well worked out and understood by the users of the test.[3] Thus, it has been shown that a test can be set up in such a way that it is positive in a large proportion of patients with a given disease when the disease is in its active form. However, if set up in such a manner, the test could give a positive result for other unrelated diseases as well as in some normal, healthy individuals. While such a test would be useful for diagnosing a particulr disease, it would give a high number of "false positives". On the other hand, if the same test is set up with a higher cut-off point it may exclude all the false positives and yet include a significant proportion of the patients with the disease in question. It is obvious, therefore, that each laboratory involved in patient-care-related laboratory medicine would have to find out the prevalence of the positivity of the relevant test first among their own normal healthy populations, second with regard to unrelated diseases and third in those diseases for which the test is useful. Such data collection for various immunological tests becomes imperative because of the known variations in these diseases as related to ethnic origin,[13] genetic variation and the variable prevalence of the infections in different geographic areas.[14] The workshops on ANA and RF held during the Fifth SEAPAL Congress at Bangkok brought out these facts very well.[7]

From this standpoint, the standardisation of ANA and RF tests for the North Indian population of Delhi was carried out at this laboratory. A cut-off point of 15 SEAPAL units/ml (1:20 dilution) excluded all unrelated and irrelevant clinical situations and detected the majority of patients with SLE and OCTD. High titres of ANA were observed both in SLE and OCTD; the ANA titre alone could not distinguish between them. The evaluation of the clinical picture and the complete auto-antibody profile, such as the estimation of anti-DNA, anti-Sm, anti-RNP, anti-SSA etc., would be helpful in distinguishing SLE from other related connective tissue diseases, and further categorising the latter.

Applied to the diagnosis of RA, the lower cut-off point of RF which would be sensitive enough to detect up to 69 per cent of the RA patients appears to be 30 IU/ml in this laboratory. However, this cut off-point would be very sensitive and therefore include some of the SLE and OCTD patients as well as patients with unrelated diseases. By increasing the cut-off point to 60 IU/ml, the RF test becomes much more specific and, to a large extent, excludes unrelated conditions. However, the sensitivity becomes as low as 53 per cent.

The study shows that by and large the ANA and RF tests give similar positivity in North Indians as they do in Western Caucasian population studies, and more or less similar cut-off points for applying these tests to clinical situations.[1,2] These findings could be very different from that seen among Mongoloid races where ANA has been reported to be negative at the time of diagnosis of SLE becoming positive much later in the course of the disease.[7] Therefore, similar studies in other races would be of interest.

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