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Antinuclear Antibody Detection Using Streptavidin-Biotin-Peroxidase Complex on HEp-2 Cell Substrate

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SUMMARY **The streptavidin-biotin-peroxidase complex (SABC) technique was compared to conventional indirect immunofluorescence (IIF) for the detection of anti-nuclear antibody (ANA) on HEp-2 cell substrate. SABC showed higher specificity and predictive value and gave more reproducible titres and clearer staining patterns than IIF in sera from a series of rheumatic disease patients. Sera from 80 patients with various types of rheumatic diseases and 20 without rheumatic disease were further tested using the SABC method. All systemic lupus erythematosus (SLE) sera were positive. The overall sensitivity was 95%, specificity 90% and predictive value 97% for rheumatic disease. The rim pattern was associated with SLE and mixed connective tissue disease. The nucleolar/homogeneous pattern was associated with scleroderma and SLE in remission. ANA titre and staining pattern have limited value in the clinical assessment of rheumatic disease; however, ANA has very high sensitivity for SLE and remains an excellent screening test.**

The immunofluorescent (IF) technique [1] has been extensively used in the detection of antinuclear antibodies (ANA). Rodent tissues were for a long time the substrate of choice. [2] However, cultured human cell lines have advantages of better sensitivity and specificity and in recent years the HEp-2 cell-line has become the preferred substrate. [3-5]

Immunocytochemical techniques using enzyme-conjugated reagents which produce colour reactions [6] have also been applied in ANA detection. They give comparable or even superior results in comparison with IF [7-9] in terms of demonstrating staining patterns which are associated with particular rheumatic diseases. [10-12] The technique has the added advantage over IF that a permanent record can be kept. The development of the avidin-biotin-complex (ABC) technique resulted in higher sensitivity [13] and it was significantly improved by substituting avidin with streptavidin which has an isoelectric point near neutral pH and therefore gives less non-specific background staining. [14]

SLE has a high prevalence in Chinese populations. [15] ANA is the principal screening test for the disease, and we considered it important to evaluate the streptavidin-biotin-peroxidase-complex (SABC) technique in comparison with conventional IF on HEp-2 cell substrate, and to assess the results of ANA testing using SABC in Hong Kong Chinese with and without systemic rheumatic disease.

MATERIALS AND METHODS

HEp-2 cell culture in chamber slides

Cells were maintained in RPMI 1640 supplemented with 10% foetal calf serum, L-glutamine 0.2 mM, penicillin 100 U/ml, streptomycin 100 μg/ml and mycostatin 20 U/ml in 50 ml tissue-culture flasks (Falcon 3013, Becton Dickinson & Co. N.J.) in an atmosphere of 5% CO_2. The cells were normally subcultured at 3 to 4 day intervals. Freshly trypsinised cells were suspended in growth medium at 1×10^5 cells/ml and one ml of this suspension was added to each

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well of the chamber slide (Lab-Tek, T/C Chambers, Miles Labs. Inc., Ill). The chamber slides were incubated in 5% CO_2 at 37°C and after 24 hours the cell monolayers were fixed in 95% ethanol for 3 minutes at room temperature and air-dried.

Indirect immuno-fluorescence (IIF) for detection of ANA

Test serum was added to the fixed HEp-2 cell monolayer and incubated for 30 minutes at room temperature. Slides were rinsed three times with phosphate buffered saline (PBS), pH 7.3, then a 1/50 dilution of fluorescein isothiocyanate (FITC) labelled goat anti-human Ig (γ-globulin fraction, Behring AG Diagnostica, Marburg) was added to the cells for 30 minutes at room temperature and the slides were again washed three times in PBS. Finally, slides were mounted in 2.5% Dabco mountant (Aldrich Chemical Co., Wis) in buffered glycerol and cells were observed by epifluorescence under blue light, 450-490 nm, using a Leitz Labolux D microscope.

SABC technique for detection of ANA

Fixed HEp-2 cell substrate was pretreated with normal sheep serum (1/5) for 20 minutes at room temperature. Test serum was added, incubated at 37°C for 30 minutes and the slide was then washed three times in Tris buffered saline (TBS), pH 7.6. A 1/100 dilution of biotinylated sheep anti-human Ig (Amersham International plc, Bucks.) was added for 30 minutes at 37°C, the slide was rinsed three times in TBS and streptavidin-biotin-peroxidase complex (SABC, Amersham International plc, Bucks.) diluted 1/50 was applied for 30 minutes at 37°C. The slide was rinsed again in TBS and developed in diaminobenzidine (DAB) 0.05% with 0.001% H_2O_2 in TBS for 5 minutes at room temperature. Finally the cell monolayer was dehydrated, cleared and mounted.

Comparison of IIF and SABC

To compare the two techniques in terms of ANA titres and patterns of nuclear staining, test sera were coded and results were read 'blindly'. Sera were screened at 1/10 dilution and positives were titred out at five-fold serial dilutions. Six sera were tested twice each to determine the reproducibility of the techniques.

Sera were tested from 47 patients and 4 normal controls; they included 11 SLE, 5 Sjögren's syndrome (SS), 3 scleroderma (Scl), 5 mixed connective issue disease (MCTD), 2 cutaneous lupus (CLE), 3 thrombocytopoenic purpura (TP) with positive ANA, 5 SLE with TP, 4 rheumatoid arthritis (RA), 9 non-rheumatic diseases and 4 normal sera.

Clinical assessment of ANA results by SABC

To study the clinical usefulness of SABC in ANA detection, we tested sera from 80 patients with rheumatic disease: 35 SLE, (including 10 cases presenting with TP); 8 CLE; 10 SS (4 overlapping with SLE, one overlapping with Scl); 4 Scl; 14 MCTD; 9 RA; Sera were also tested from 20 individuals without rheumatic disease and 10 sera from healthy adults were used as 'normal controls'.

The earliest serum available from each patient within the study period was retrieved from the freezer files. In the case of SLE, only those newly diagnosed in the study period were selected and, if available, a second serum taken after 5 to 7 months was included (paired sera were available in 11). All sera were coded and tested 'blindly' for ANA by the SABC method. Sera were first tested at a screening dilution of 1/10. Positives were then titred out at serial five-fold dilutions.

Clinical activity score

Each patient having SLE, MCTD and SS/SLE was given a 'clinical activity score', ranging from 0-3, when the serum was taken. [16] A score of 0 indicated complete remission, while scores of 1 to 3 indicated disease activities of increasing magnitudes.

Stastical methods

The correlation of results between IIF and SABC was analysed by Kappa statistics. [17] The comparison of titres was analysed by the linear regression test. Fisher's exact test was used for the rest of the statistical analysis.

RESULTS

Comparison of IIF and SABC

Results of ANA assays on 51 sera, tested at 1/10 dilution, are summarised in Table 1. In terms of

Table 1. Correlation of positive and negative ANA results given by IIF and SABC

		SABC		
		+	−	Total
IIF	+	38	7	45
	−	1	5	6
	Total	39	12	51

(K=0.51)

negativity and positivity the two techniques correlated reasonably well (K = 0.51) but IIF was slightly more sensitive at the screening dilution employed.

Sera from patients with rheumatic disease and a control group (non- rheumatic disease and normals) were tested by both techniques and the results are shown in Table 2. The sensitivity for rheumatic disease was 97% by both techniques. On the other hand, specificity was considerably higher by SABC (85%) than by IIF (38%). The positive predictive value of ANA using SABC was also higher (95%) than using IIF (82%).

In terms of titres, the two techniques correlate well (r = 0.62). However, of the 37 sera positive by both techniques, only 14 (36%) gave the same titre by both methods. In the remainder, discrepancies were from 1 to 3 tube dilutions.

Reproducibility was compared by testing 6 sera on two occasions. SABC gave full correspondence of negativity and positivity, but one serum was positive with IIF in the first (titre = 10) and negative in the second assay. Titre discrepancies of 1 and 2 tube dilutions were noted in 2 sera with IIF. One serum showed a 1 tube discrepancy with SABC. Thus reproducibility was apparently better using SABC.

In respect of nuclear staining patterns, the nucleolar pattern was observed with SABC but not with the IIF technique. Pretreatment of the HEp-2 cells with RNase A (1 g/ml) prior to fixation abolished the nucleolar pattern, confirming that the nucleolar staining was specific for RNA. The nucleolar pattern could be readily demonstrated by IIF, however, if cells were fixed in acetone instead of ethanol.

Clinical assessment of ANA results using SABC

Table 3 shows the proportion

Table 2. Comparison of IIF and SABC in ANA detection in rheumatic disease.

	IIF			SABC		
	+	–	Total	+	–	Total
Rheumatic disease	37	1	38	37	1	38
Controls	8	5	13	2	11	13
Total	45	6	51	39	12	51
Sensitivity	37/38 x 100 = 97%			37/38 x 100 = 97%		
Specificity	5/13 x 100 = 38%			11/13 x 100 = 85%		
Predictive value	37/45 x 100 = 82%			37/39 x 100 = 95%		

Table 3. ANA results in different disease categories

Diagnosis	No.	+ve	–ve	% (+ve)
SLE	25	25	0	100%
SS	10	10	0	100%
SCL	4	4	0	100%
CLE	8	8	0	100%
SLE/TP	10	10	0	100%
MCTD	14	13	1	93%
RA	9	6	3	67%

ANA performed by SABC method, with serum screening dilution of 1/10.

Table 4. ANA result in rheumatic and non–rheumatic disease

Disease	+	–	Total
Rheumatic disease	76	4	80
Non–rheumatic disease	2	18	20
Total	78	22	100
Sensitivity	76/80 x 100 = 95%		
Specificity	18/20 x 100 = 90%		
Predictive value	76/78 x 100 = 97%		

ANA performed by SABC method, with serum screening dilution of 1/10.

of patients in each category of rheumatic disease who were ANA positive. In most groups positivity was 100% and the lowest proportion was 67% in RA. Table 4 summarises the ANA positivity in the broad groups of rheumatic and non-rheumatic diseases showing that the assay gives high sensitivity (95%) and specificity (90%) and a very high positive predictive value (97%).

Six nuclear staining patterns were observed, namely, homogeneous, rim, fine speckled, coarse speckled, nucleolar and centromeric (ANA reference sera from CDC, Atlanta tested in parallel showed the appropriate patterns for each sera.)

Fine speckled and coarse speckled were the predominant patterns. The rim and homogeneous patterns never occurred singly and mixed patterns were commonly found. The components of a mixed pattern would often titre out at different dilutions in which case the highest titre was recorded as the ANA titre. Mixed patterns were commonly found in SLE and MCTD. The rim pattern was significantly associated with these subgroups ($P < 0.05$) and mixed patterns of three components were seen in these two subgroups. The rim pattern always had a lower titre (10-250) than its associated pattern(s).

A mixed nucleolar/homogeneous pattern was only observed in Scl ($P < 0.01$). Nucleolar patterns were observed in 3/7 cases of SLE with TP at higher titres (> 50) while low titre (10-50) nucleolar patterns were also seen in non-rheumatic diseases.

ANA titres were generally higher in SS than in the other rheumatic diseases ($P < 0.001$) being > 6,250 in all 6 SS patients studied.

Correlation with clinical activity

Generally speaking the ANA patterns, whether single or mixed, were not correlated with levels of disease activity (Fig. 1). However,

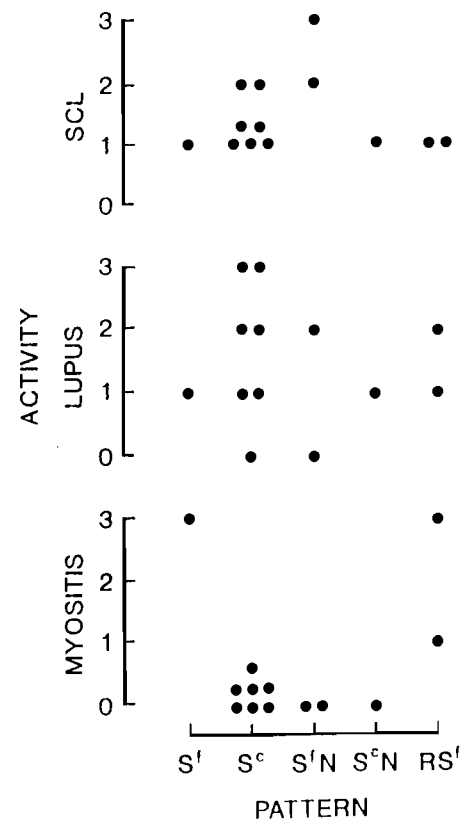

Fig. 1 Association of ANA patterns and disease activity scores in MCTD (n = 13). S^f, fine speckled : S^c, scores speckled : N, nucleolar : R, rim.

Table 5. Sequential changes in patterns and titres of paired consecutive sera from individual patient with SLE

	Titre		Pattern		Score	
	1st	2nd	1st	2nd	1st	2nd
Rising	50	1250	S^f	R/H	2	2
	250	156250	S^c	S^c	3	3
Steady	1250	1250	R/H	S^f/N	3	1
	6250	6250	R/S^f	R/S^f/N	2	1
	6250	6250	R/S^f/N	S^c	3	1
	31250	31250	S^f	S^c	3	2
	31250	31250	S^f	S^f	2	2
Falling	250	–ve	N	–ve	2	0
	1250	250	R/S^f	N/H	2	0
	6250	1250	R/S^f	N/H	2	0
	31250	6250	R/S^f/N	R/S^f	3	1

Remarks: S^f: fine speckled; S^c: coarse speckled
H: homogeneous; N: nucleolar; R: rim

in MCTD, inactivity of the myositis component was associated with the coarse speckled ANA pattern, and the speckled/nucleolar pattern.

A wide range of ANA titres was seen for each activity score, so that the mean log titres for each score showed little difference. Eleven pairs of sera from patients with SLE were analysed, the mean duration between first and second sera being 6 months. The two sera of each pair were always tested at the same time. The findings are summarised in Table 5. A lower titre in the second serum always corresponded to decreased clinical activity. Unchanged titres corresponded to decreased or unchanged clinical activity score. Two sera which showed increased titres corresponded to no change in clinical score. Thus, although titre changes did not strictly correlate with clinical activity, there was no gross discordance between the two.

Ten patients with SLE who died during the course of the study and who might have represented a poor prognosis group were compared with 25 other SLE patients. No significant difference was found in terms of ANA patterns or titres.

DISCUSSION

In both IIF and SABC it is essential to maximise the ratio of specific staining intensity to non-specific background staining intensity ("signal to noise ratio"). In the SABC method used in this study, blockage of endogenous enzyme activity was not necessary because endogenous peroxidase activity in the HEp-2 cells was low, while pre-treatment with normal sheep serum was necessary to block tissue ligands with affinity for the labelled sheep immunoglobulin. Objectively, "signal to noise ratio" was higher in SABC resulting in superior clarity of ANA patterns.

The finding that test sera showed the nucleolar pattern with SABC and not with IIF was unexpected. The difference was shown to be related to the method of fixation: using IIF on HEp-2 cells fixed in acetone, the nucleolar pattern was readily demonstrable by both clinical samples and the CDC reference serum.

Titres by the two techniques correlated fairly well ($r = 0.62$), the correlation coefficient being a little lower than in a similar study by Fritzler *et al* ($r = 0.77$).[18] With respect to rheuamtic diseases, the two methods had the same diagnostic sensitivity (95%) but specificity and predictive value were higher using SABC.

The ANA test performed on HEp-2 cells has a higher sensitivity than ANA done on rodent tissue substrates. Moreover recent experience in Hong Kong, using IF on HEp-2 substrate in testing a large series of well documented SLE cases, has confirmed the very high sensitivity for SLE (98%). However, specificity and predictive value for SLE are low, so the test must be used essentially as a screening test for this disease.

Previous studies have shown that ANA negative SLE (as detected on rodent tissues) is characterised by cutaneous involvement, arthritis and a mild form of renal disease.[19] In this series, all patients with CLE had a positive ANA, suggesting that HEp-2 cells may be a better substrate to detect ANA in this disease subset.

This study has confirmed our experience that the recognition of ANA nuclear staining patterns is of only limited value in diagnosis, prognosis or assessment of disease activity. An exception is the centromere pattern of staining which is known to be specific for Scl and the CREST syndrome. The association of rim or rim/homogeneous patterns with SLE has been previously reported.[10,11] In our present study the rim pattern was associated with SLE and MCTD. We observed the nucleolar/homogeneous pattern in 2 of 4 patients with Scl and this association with Scl supports the finding of Kallenberg *et al.*[12] The coarse speckled pattern had a higher frequency in SLE having clinical scores of 2 and 3, and may indicate higher disease activity. In MCTD the speckled (coarse or fine)/nucleolar pattern was associated with inactive myositis (Fig. 1). The mean log titres were found to increase with activity score in SLE (but not in MCTD or SS); this was similar to the finding of Husain.[20] However, a wide range of titres was observed at each level of activity so the titre of any single specimen was of little help in assessing disease activity. The possible correlation of ANA titres with disease activity in SLE has been a matter of controversy,[21-24] but it is now generally recognised that the initial ANA titre is of little prognostic value. In the present study a falling or steady titre corresponded to alleviation of disease. To a large extent the problem lies with the inherent lack of precision of the ANA assay as usually performed on a single substrate and the result expressed simply as a titre. Both test precision and concordance between laboratories can be greatly improved by using an international reference standard to create a standard curve for each assay and expressing the results in units.[25]

In conclusion, the SABC technique, in comparison with IIF, gave superior clarity of ANA nuclear staining patterns, higher specificity and predictive value for rheumatic diseases, and better reproducibility. Titres by the two techniques correlated well. SABC had the added advantage that slides could be stored for long periods. The important drawbacks of SABC were its higher reagent cost (five times more than IIF) and the longer processing time required (one hour more than IIF).

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