Use of cultured epithelial cells, including keratinocytes, for the detection of antinuclear antibodies

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SUMMARY The use of cultured human epithelial cells as a substrate for the detection of antinuclear antibodies (ANAs) improves the sensitivity of detection above the standard substrates used. Standard antisera have been screened against a series of epithelial cell lines, including keratinocytes. Selected cells have then been used to detect ANAs in 78 patients with discoid lupus erythematosus.

Key words: discoid lupus erythematosus.

Antinuclear antibodies (ANAs) are routinely detected by the indirect immunofluorescent staining of sections of animal tissues, commonly rodent liver. The detection of ANAs and their titre and pattern depend on the substrate used.¹ Human cell lines show greater sensitivity than standard substrates and in addition different patterns of ANA staining can be detected, such as anticentromere and anti-centriolar patterns, in patients with scleroderma.^{2 3} We have therefore screened standard sera⁴ with defined patterns of ANA reactivity on mouse liver against a number of epithelial cell lines and identified those giving greatest sensitivity and reproducibility. Keratinocytes appear to be a target for ANA binding in cutaneous lupus erythematosus,⁵ and as it is now possible to culture normal human keratinocytes⁶ these were included in the series of cells tested.

ANAs are detected with conventional substrates in only 20-30% of patients with discoid lupus erythematosus (DLE).⁷ We have screened sera from patients with DLE diagnosed by clinical, histological, and immunocytochemical criteria, on the selected cell lines to determine whether the use of these substrates increases the detection of ANAs in DLE.

Materials and methods

CELL CULTURE SYSTEMS

Normal human keratinocytes were prepared as a single cell suspension by treatment of keratotomed adult breast skin (from breast reductions) with 0.25% trypsin at 37°C for two to three hours. The cells were seeded into 5 cm dishes at a density of 10 000/cm² in the presence of a feeder layer of irradiated 3T3 cells using the medium described by Wu *et al.*⁸ The cells formed confluent cultures two to three weeks after seeding and were fixed in methanol: acetone (1:1) for five minutes.

The cell lines (see Table 1) were cultured on multiwell slides using Dulbecco's minimal essential medium and 10% fetal calf serum and fixed in methanol:acetone 1:1. All samples were stored in

Table 1 *Origin of cell lines*

Cell line	Origin of cell line
SVK14	SV40 transformed keratinocytes ¹⁷
HEP ₂	Laryngeal carcinoma line
TR146	Human squamous cell carcinoma line ¹⁸
CAMA1	Human breast carcinoma line ¹⁹
T47D	
MDA157	
PsF1	
BEK	Psoriatic fibroblasts derived from skin biopsies of involved skin
	Bovine embryonal kidney

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Table 2 Standard sera (A): highest titre detected on each substrate

Cell line	Standard sera* (group A)				
	Diffuse	Speckled	Peripheral	Nucleolar	Thready
HEp ₂	1/2560	>1/10 240	1/40	>1/10 240	1/160
CK†	1/320	1/320	1/40	1/80	1/40
SvK14	1/2560	1/2560	1/320	>1/10 240	1/640
TR146	1/160	1/320	1/80	>1/10 240	1/320
CAMA1	1/10 240	1/2560	1/320	1/320	1/320
T47D	1/10 240	1/10 240	1/10 240	1/10 240	1/640
MDA157	1/10 240	1/10 240	1/80	>1/10 240	1/640
PsF1	1/640	1/2560	1/80	>1/10 240	1/320
BEK	1/640	1/2560	1/40	>1/10 240	1/320

*Kind gift of Dr Burnham.

†CK=cultured keratinocytes.

liquid nitrogen until used. Indirect immunofluorescence staining was performed using standard techniques with fluorescein isothiocyanate labelled goat antihuman IgG and IgM (DAKO).

STANDARD SERA

Group A. Five standard sera (kind gift of Dr Burnham) were used against all cell lines in dilutions from 1 in 4 to 1 in 20 560. They had shown a defined pattern of reactivity on mouse liver or spleen imprint that was diffuse (D), speckled (S), peripheral (P), nucleolar (N), or thready (T).

Group B. Substrates were also tested with standard antisera to diffuse, speckled, and nucleolar patterns kindly provided by the CDC, Atlanta.

TEST SERA

Serum samples were tested from 78 patients with discoid lupus erythematosus diagnosed by clinical, histopathological, and immunopathological criteria. These patients therefore had chronic cutaneous lesions but no systemic manifestations of lupus erythematosus, no increased DNA binding capacity, and no extractable nuclear antigen reaction. No patients were receiving systemic treatment, apart from six who were receiving mepacrine. Clinical features were analysed according to the presence of photosensitivity, Raynaud's phenomenon, chilblains, and skin type.⁸ These data will form the basis of a separate report (S Mayou). Sera from 30 controls (healthy blood donors aged 18–65) were also studied. The results were assessed according to the presence of antinuclear antibodies and their titre and the pattern of staining, using the terminology of Burnham.⁹ Samples were read blind by the same observers (TZ, SB). Each sample was tested repeatedly; the pattern of staining was consistent, although where the titre varied, the mean value was taken.

Results

All the human cell lines demonstrated the ANAs of the standard sera, but the titres and patterns obtained were dependent on the substrate used.

VARIATION IN TITRE

The titre of ANAs obtained using the reference antisera varied with the substrate as shown in Table 2. There was a fourfold to 256-fold variation in titre of the individual antisera with different substrates (Table 2). The greatest titre variation was seen with peripheral and nucleolar antibodies. T47D was the most sensitive for the peripheral pattern of ANAs. The sensitivity of the other cell lines was similar in many cases to that of HEp₂ cells. Interestingly, cultured keratinocytes provided a less sensitive substrate than most of the keratinocyte derived lines, even the closely related SvK14 cells (Table 3).

VARIATION IN PATTERN

The pattern of ANAs also varied with the substrate (Table 3). The diversity of pattern found in the diffuse ANA group probably reflected increased sensitivity of the cultured cells at high dilution, which allowed the visualisation of other antibodies normally masked by the diffuse staining. This was seen with both sets of standard sera (Tables 4 and 5). In the American standard reference sera

Table 3 Standard sera (A): variation in titre of ANA with substrate

Pattern	Lowest titre	Highest titre
Diffuse	160	10 240
Speckled	320	10 240
Peripheral	40	10 240
Nucleolar	80	10 240
Thready	40	640

Table 4 Standard sera (A): variation in pattern of ANAs with substrate

Cell line	Standard sera—patterns detected				
	Diffuse	Speckled	Peripheral	Nucleolar	Thready
HEp ₂	D/S/P	S/P/T	P	N/S	T/P/S
CK	D/—/P	S/D	P/D	N	Not done
SvK14	D/S/P	S/P	P/D	N	T/S
TR146	D/S/P	S/P	P	N	T/S
CAMA1	—/S/P	S/D	P	N	T/P
T47D	D/S/P	S/D/P	P	N	T
MDA157	—/S	S/P	P/D	N	T
PsF1	D/S/P	S	P/D	N	T
BEK	—/P	S/P	P	N	T

(group B) a distinct shift in pattern of diffuse staining was seen on dilution to peripheral staining (Table 5). Although most substrates recognised the expected pattern, multiple patterns were often seen,

particularly in the diffuse and speckled group (A sera). The patterns were characterised according to the system of Burnham⁹ and are shown in the figures against SvK14 cells as this has not previously been published as a substrate for ANA testing (Fig. 1).

Table 5 Standard sera group B—American national reference sera: variation in pattern with substrate on dilution

Cell line	HEp ₂	TR146	SvK14	CK
Diffuse	D	D	D	D
on dilution	P	P	P	P
Speckled	S	S	S	S
Nucleolar	N	N	N	N

DETECTION OF ANAS IN SERA OF PATIENTS WITH DLE (Tables 6 and 7)

T47D cells were selected because of their sensitivity for peripheral antibodies and HEp₂ cells as these are the most commonly used. Cultured keratinocytes and sections of mouse liver were also used for testing the DLE sera.

Fifty four per cent of patients with DLE had positive ANAs on one or more substrate but only 20% on mouse liver alone. The cell lines had different sensitivities. The maximal yield with one substrate alone was with HEp₂ cells (45%), while T47D cells detected 37%. The human keratinocytes were less sensitive, detecting only 22% and contributing only one positive that was not detected by another substrate. Forty seven per cent of the positive sera showed a speckled pattern which varied little on dilution. The control sera gave no positive results on mouse liver and 17% positive with HEp₂ cells at a titre of 1/10 and 3% at a titre of 1/40. The patterns were particulate (speckled and thready).

Table 6 Patterns of ANAs in 78 patients with DLE

Pattern	No (%)
Speckled	37 (47)
Peripheral	15 (19)
Diffuse	8 (10)
Thready	9 (11)
Nucleolar	3 (4)

Table 7 ANA detection in DLE is substrate dependent

Substrate	No of positive sera (%)
All substrates	42 (54)
Mouse liver	16 (20)
All cell lines	39 (50)
Cell lines—additional positives	
HEp ₂ —total positives	35 (45)
HEp ₂ —alone	7 (9)
T47D—total positives	29 (37)
T47D—alone	2 (3)
Keratinocytes—total positives	17 (22)
Keratinocytes—alone	1 (1)

Discussion

The use of nuclear fluorescent patterns of ANAs as immunological markers for subsets of connective tissue diseases has led to a complex system of classification.⁹ The nuclear fluorescent patterns are described as non-particulate peripheral and homogeneous (diffuse) staining and particulate staining, including speckled, nucleolar, and thready patterns. Disease associated patterns have been reported for speckled (calcinosis, Raynaud's phenomenon, oesophageal dysmotility, sclerodactyly, telangiect-

Fig. 1a

Fig. 1b

Fig. 1c

Fig. 1d

Fig. 1 ANA pattern of SvK14 cells. *a. Homogeneous. b. Peripheral. c. Speckled. d. Nucleolar (multiple nucleoli).*

tasia (CREST) syndrome)¹⁰ and nucleolar (systemic sclerosis) or peripheral (systemic lupus erythematosus).¹ Indirect immunofluorescent techniques show quantitative and qualitative differences between substrates.¹¹ The classical substrate, mouse liver, does not show the same sensitivity as human spleen cells,¹² presumably because there is species variation in antigen structure or presentation. Cultured cell lines have been used as substances for ANAs to eliminate the problems of tissue variation and to increase sensitivity and reproducibility. Intracellular detail is also easier to detect in the flat spread out nuclei of cell monolayers. In addition, a high percentage of cells divide in culture, and nuclear antigens which are cycle dependent, such as the proliferating cell nuclear antigen,¹³ can be detected more readily.

We have shown that all the human cell lines used were suitable for the detection of ANAs but confirmed that the titre and pattern of ANA staining were markedly substrate dependent. HEP₂ cells and T47D were the most sensitive for detection of ANAs in standard sera, T47D showing particularly high expression of peripheral antinuclear staining. A number of different staining patterns were shown by the same sera, confirming that patients may produce autoantibodies to more than one nuclear antigen.¹ SvK14 cells were more sensitive than primary keratinocytes, which must reflect altered nucleoprotein antigenicity following transformation.

The use of HEP₂ and T47D cells as substrates for testing DLE sera increased the number of positive sera compared with mouse liver, but the use of normal human keratinocytes conferred small additional benefit (1/78 positive patients). The number of DLE patients with ANAs was less than in the study of Wermuth *et al* of DLE/subacute LE,¹⁴ but this was because we excluded patients with subacute LE. The use of multiple substrates for screening was essential as different substrates detected different positive ANAs. The combined substrates detected positive ANAs in 54% of patients compared with 20% on mouse liver alone. There was no correlation between positive ANAs and photosensitivity, chilblains, Raynaud's phenomenon, arthralgia, skin type, or medication (Mayou, unpublished data). Screening of patients with DLE on multiple human cell lines combined with longitudinal studies will be necessary to determine the clinical and prognostic significance of these findings.

The multiple patterns of antibody reactivity found using cell lines may reflect polyclonal B cell stimulation in patients with LE.¹⁵ Distinct patterns of reactivity on different substrates can be shown by immunodiffusion to be identical antigens, e.g., anti-Ro and SSA antibodies.¹⁶ The variation in pattern

with substrate emphasises the importance of biochemical characterisation of antigenic sites and the need for molecular biology techniques to clarify the specificity of the patterns. As these techniques become more widely available comparison of different laboratory results will be easier.

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