Ineffectiveness of rat liver tissues in the screening of connective tissue disease

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ABSTRACT

Objective: To assess the effectiveness of using rat liver tissues (RLT) for the screening of connective tissue disease (CTD).

Methods: Results of patient samples, submitted to the Clinical Immunology Laboratory, Birmingham Heartlands Hospital, Birmingham, United Kingdom for investigation of CTD between 2001 and 2002, were analyzed. Positive results for anti-double stranded DNA (dsDNA) antibodies and anti-extractable nuclear antigen (ENA) antibodies were correlated with the results of the corresponding anti-nuclear antibodies (ANA), obtained by indirect immunofluorescence (IIF) using RLT. In the second part of the study samples that were previously tested positive for anti-ENA or anti-dsDNA antibodies, were investigated prospectively for ANA using both RLT and human epithelial (Hep-2) cell line.

Results: The IIF method employing RLT for screening of CTD, failed to detect ANA patterns from 45% and 25% of patient samples know to contain antibodies to dsDNA and ENA. The anti-dsDNA antibodies that failed to be detected by the RLT were of low avidity and their clinical significance is unknown. In contrast, the antibodies to ENA were mostly directed against the Ro antigen and a clear marker of CTD. Hep-2 cell line enhanced the detection rate of anti-ENA antibodies, particularly those against the Ro antigen. In contrast, and like RLT, Hep-2 cell line failed to detect the low avidity anti-dsDNA antibodies.

Conclusion: The present study has clearly shown that RLT are ineffective for screening of CTD. It is recommended that laboratories, which are still using these tissues, should consider replacing them with the Hep-2 cell line.

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Antinuclear antibody (ANA) testing is widely used for the diagnosis, prognosis and monitoring of connective tissue disease (CTD) including systemic lupus erythematosus (SLE), Sjogren's syndrome (SJS), mixed connective tissue disease (MCTD), systemic sclerosis (SS), CREST (calcinosis, Raynaud's, esophageal dysmotility, sclerodactyly, and telangiectasia) syndrome, polymyositis and dermatomyositis.[1,2] Traditionally, the initial screening for CTD was carried out by indirect immunofluorescence (IIF) method using animal tissues or human-derived cells. The patterns of ANA staining are noted and used to further direct investigation for more specific ANA using a variety of different systems.[1-4] Such antibodies include those directed against the native form of DNA [double stranded DNA (dsDNA)] and the saline extractable nuclear antigens (ENA). The latter antigens include Ro (SS-A), La (SS-B), smith antigen (Sm), ribonucleic protein (RNP) and Scl-70 (Topoisomerase-1).[2,5] Some of the ANA patterns are diagnostic on their own rights, for example nucleolar patterns are associated with SS and the discrete speckled pattern on dividing cells (centromere) are associated with CREST syndrome.[6,7] However, the majorities of patterns encountered in every day work suggest a diagnosis and are used to further direct the investigation into

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particular antibodies. Such patterns include the homogeneous and the nuclear rim, which are associated with SLE and drug-induced SLE, and are linked with anti-dsDNA and histones-antibodies. Speckled ANA patterns that are associated with SJS, SLE, MCTD and SS are linked to antibodies against ENA antigens (Ro, La, Sm, RNP and Scl-70).[2,3] For a summary of the different ANA patterns and their association with individual nuclear antigens and different CTD (**Table 1**). Since the diagnosis and subsequent investigations of CTD depend, in general, on the initial screening results, it is vital that any screening method used must show a good sensitivity for the detection of all the different nuclear patterns and their associated antigens. In the past, different tissues and cell-lines have been used for ANA screening including those derived from animals (dogs, mice, monkeys and rats) and human.[2,8] However, rat liver tissues and the human-derived cell line (Hep-2) have been used widely and extensively in clinical immunology laboratories. Rat liver tissues are generally regarded inferiors to the Hep-2 cell line for a number of reasons. Firstly rat liver cells are not dividing and therefore the centromere pattern can not be detected on these tissues. Secondly, ENA leach out more easily from the liver cells and thirdly, some of the ENA, particularly the Ro antigens, are either absent or expressed in a very low amount.[2] In contrast, Hep-2 cells are dividing with large nuclei and nucleoli and hence have large amount of nuclear antigens, including the Ro antigen.[9] In spite of these advantages of Hep-2 cell line, many clinical immunology laboratories continue to use rat liver tissues for their initial screening of CTD. This could be explained by, firstly, the lack of strong published data showing conclusively the ineffectiveness of these tissues in the screening of CTD and, secondly, by the cheap cost of such tissues. However, to maintain using rat liver tissues in the initial screening, it is vital that these tissues are proven to be capable of detecting all the anti-nuclear antibodies that are manifested by the different CTD. I have therefore undertaken the present investigation to probe the effectiveness of using rat liver tissues used as an initial screen of CTD. I have retrospectively compared the various anti-nuclear antibodies, namely those against dsDNA and ENA, with the ANA-results obtained by IIF using rat liver tissues from cases where all of these tests were carried out simultaneously. In addition, rat liver tissues were compared with Hep-2 cell line for the detection of ANA patterns and hence for screening for CTD. As far as I am aware from the literature, this type of investigation has not been conducted previously.

Table 1 - Antinuclear antibodies-pattern and their association with different nuclear antigens and associated connective tissue disease.

ANA patterns	Corresponding antigens	Associated CTD
Homogenous	dsDNA, Histones	SLE/drug induced SLE
Peripheral	dsDNA	SLE
Speckled	Sm RNP Ro/La Scl-70	SLE SLE/MCTD SJS/SLE SS
Discrete speckles	Centromere	CREST
Nuclear membrane		PBC
Nuclear Pores		PBC
Nucleolar	Nucleolar RNA PM-Scl	SS Polymyositis-SS
Nuclear dots	Sp100 (Nsp1), Colin p80	SJS, PBC

ANA - antinuclear antibodies, CTD - connective tissue disease,
dsDNA - double stranded DNA,
Sm - Smith antigen, RNP - ribonucleic protein,
Scl-70 - scleroderma-70, RNA - ribonucleic acid,
PM-Scl - polymyositis-scleroderma,
SLE - systemic lupus erythematosus,
MCTD - mixed connective tissue disease, SJS - Sjögren's syndrome,
SS - systemic sclerosis, CREST - Calcinosis, Raynaud's, Esophageal Dysmotility, Sclerodactyly and Telangiectasis,
PBC - primary biliary cirrhosis

Methods. Collected samples submitted to the Clinical Immunology Laboratory, Birmingham Heartlands Hospital, Birmingham, United Kingdom for investigation of possible CTD between the periods of 2001 and 2002 were retrospectively analyzed. Those found to have been simultaneously tested for anti-dsDNA and anti-ENA antibodies were included in the present study. Some of these results originated from samples of the same patients tested repeatedly over the above period. In the laboratory, testing for ANA, dsDNA and ENA were carried out using indirect immunofluorescence (IIF) using rat liver tissues, an enzyme-linked immunosorbent assay (ELISA) and the counter current immunoelectrophoresis (CCIE) methods. Positive results for dsDNA and ENA antibodies were then compared with that of the corresponding ANA-results. Screening for dsDNA antibodies was carried out initially by an ELISA method and positive samples are then further tested by more clinically relevant method called Crithidia Luciliae. Therefore in addition, dsDNA results (by ELISA) were compared with that obtained by Crithidia Luciliae. This was carried out in order to find out at what levels of positive anti-dsDNA antibodies by

ELISA start to be detected by Crithidia Luciliae and thus give an indication of the quality of the antibodies.

Parallel testing for antinuclear antibody using rat liver tissues and Hep-2 cell line. In order to compare the efficiency of rat liver tissues and different preparations of Hep-2 cell line for screening of CTD, a number of samples; know to contain antibodies to different ENA and to dsDNA, were prospectively tested for ANA patterns on these 2 tissues.

Results. ***Correlation between dsDNA and ANA-results.*** Initially, positive dsDNA results, obtained by the ELISA method, were compared with the ANA-results obtained by the IIF using rat liver tissues. Between the period of 2001 and 2002, 214 samples tested positive for anti-dsDNA antibodies. When these latter results were compared with the ANA-results, 96 (45%) had correspondingly negative ANA-results. These findings indicate that using rat liver tissues could potentially miss out 45% of samples with positive anti-dsDNA antibodies if used as a first line of screening for CTD. Next, I investigated the nature and significance of these anti-dsDNA antibodies, particularly those with correspondingly negative ANA-results.

Comparison between anti-dsDNA antibodies obtained by ELISA and the Crithidia Luciliae methods. In this section, I have initially compared positive anti-dsDNA antibody results obtained by the ELISA method with that obtained by the Crithidia Luciliae. Two hundred and forty-two samples tested positive by the ELISA method for anti-dsDNA antibodies. However, 170 samples (70%) tested negative and a further 12% tested weakly positive by the Crithidia Luciliae method. In contrast, only 44 samples (18%) of the total samples tested positive by the latter method. Of the 170 samples tested negatively by Crithidia Luciliae, approximately 119 (70%) had anti-dsDNA antibody values by the ELISA method of 104 $\pm$ 8.8 IU/ml. In contrast, in weak positive and positive samples, the values of anti-dsDNA antibodies were 131 $\pm$ 13 and 507 $\pm$ 50 IU/ml. These findings indicate that only positive samples tested by the ELISA method with values greater than 100 IU/ml start to be detected by the Crithidia Luciliae method. It would be reasonable therefore to suggest that the initial screening for anti-dsDNA antibodies is carried out by ELISA and that any samples with values greater than 100 IU/ml are investigated further by the Crithidia Luciliae method, or if there is a strong clinical indication. Finally, samples with positive values for anti-dsDNA antibodies (by ELISA), but with correspondingly negative ANA-results on rat liver tissues, all had negative results by the Crithidia Luciliae method.

Correlation between results of antibodies to the extractable nuclear antigens and antinuclear antibody results. Antibodies to the extractable nuclear antigens are assessed by the counter current immunoelectrophoresis (CCIP) method using thymus extract as a source of total ENA and run in parallel with one or more of the purified individual antigens. The most frequent antibodies examined were the Ro, the La and to some extent the RNP (**Table 2**). Initially, positive ENA results were compared with the corresponding ANA-results. Out of 140 ENA-positive samples, 35 (25%) had correspondingly negative ANA-results. As with dsDNA results, these findings indicate that the present ANA screening method could potentially miss out approximately 25% of patients with ENA-linked CTD. In order to find out a possible explanation for the negative ANA-results, further analysis of the individual antibodies to ENA, namely the Ro the La and the RNP were carried out. Out of the 35 ENA positive ANA negative samples, 23 were also tested in parallel for Ro and La antibodies. Examining these results revealed that the majority of these samples (n=20) tested positive for Ro, but negative for La. The other 3 samples were positive for both Ro and La. These results clearly indicate that the ANA screening method using rat liver tissues fails to pick up positive anti-Ro antibodies. Unlike anti-Ro, the presence of anti-RNP antibodies, even when occurring alone (n=7), correlated well with positive ANA-results. Since anti-Ro antibodies are strongly associated with Sjogren's syndrome, occurring in approximately 70-90 of patients,[14] I next investigated ENA-results obtained from samples, which had clinical diagnosis label of Sjögren's syndrome. There were 31 samples, 14 (45%) had negative ANA-results. Eleven of these samples had positive results for Ro, but negative results for La. The other three had positive results for both antibodies. Comparison between ANA-results obtained by IIF method using rat liver tissues and Hep-2 cell line. Finally, samples known to contain antibodies to ENA were examined prospectively for ANA using rat liver tissues and Hep-2 cell line. In total 21 samples were examined. As shown in **Table 3**, only 52% of these samples tested positively on rat liver tissues. As expected, the great majority of the ANA negative samples were those with anti-Ro antibodies. However, Hep-2 cell line increased the rate of ANA detection, from 52% on rat liver tissues, to greater than 80%. Moreover, the rate of detection of samples with Ro antibodies was increased, from a mere 16% on rat liver tissues, to greater than 67% on Hep-2 cell line. In addition, Hep-2 cell line detected additional ANA patterns that could not be detected by the rat liver tissues including the centromere patterns (results not shown). In contrast to ENA, samples with positive

anti-dsDNA antibodies (by ELISA; n=15), that had previously tested negatively for ANA on rat liver tissues, tested negatively for ANA on both rat liver tissues and Hep-2 cell line.

Discussion. Antinuclear antibody testing plays a pivotal role in the diagnosis, prognosis and monitoring of CTD. Initial screening is carried out by IIF using animal tissues, or human derived cells and further investigations are carried out for specific anti-nuclear antibodies on positive samples.[1-4] In the present study, I have investigated the effectiveness of the IIF- screening method, using rat liver tissues, for the detection of CTD. The study clearly shows that approximately 45% of patients with positive anti-dsDNA antibodies could potentially be missed out by such screening method. These anti-dsDNA antibodies, in samples with negative ANA, can not be attributed to false detection of contaminating anti-single stranded-DNA antibodies, since the present ELISA method detects specifically anti-dsDNA antibodies. However, the results strongly suggest that these antibodies are of low avidity, which although can bind to purified dsDNA in an ELISA system, bind poorly to dsDNA in situ (rat liver tissue and Crithidia Luciliae) and could be subsequently washed off easily from slides. It is equally possible that the affinity of the rat liver tissues and Crithidia Luciliae cells for these low avidity antibodies is low and therefore less binding occurs. Anti-dsDNA antibodies are associated with SLE and their presence is used for the diagnosis, prognosis and monitoring of the disease.[13-15] High avidity antibodies are associated with complement consumption and glomerulonephritis[13] and both the ELISA and the Crithidia Luciliae methods detect such antibodies. In the present study, with exception of 2, all samples (53) with clinical diagnosis of SLE had positive anti-dsDNA antibodies (by ELISA) and correspondingly positive ANA-results. Out of the samples 33 showed positive anti-dsDNA antibodies by Crithidia Luciliae and 25 (75%) were associated with decreased complement components. In contrast, virtually all samples with positive anti-dsDNA antibodies by ELISA, but with negative ANA-results by rat liver tissues, had non-specific clinical signs and symptoms. Moreover, all other immunological tests performed on these samples were normal including the complement components (C3/C4) and therefore the clinical significance of these antibodies is unknown. However, since these antibodies were associated with clinical signs and symptoms that could be manifested by any of the systemic CTD including SLE, it is tempting to speculate that, at least in some cases, these antibodies could be the first manifestation of CTD and that, with time, these antibodies could undergo maturation and hypermutation to the high avidity anti-dsDNA antibodies leading eventually to the full manifestation of the SLE disease. The process of maturation and hypermutation of low avidity anti-dsDNA antibodies to high avidity is well documented in SLE.[16,17] The present study also revealed that the current screening method is also

Table 2 - The frequency of specific anti-ENA antibodies detected in 140 ENA positive samples.

Antibodies	Total number of ENA positive samples examined	Samples found to be positive n (%)
Ro	119	93 (78)
La	94	46 (49)
RNP	39	17 (39)
Scl-70	14	2 (14)
Sm	2	1 (50)

In total, 140 samples with positive antibodies to the total extractable nuclear antigens (ENA) were recorded over the one-year period. Of these, 114 samples were found to contain antibodies to specific ENA. Scl-70 - scleroderma-70, Sm - Smith antigen, RNP - ribonucleic protein

Table 3 - Comparison between Rat liver tissues and Hep-2 cell line for the detection of ANA.

Samples tested positive by CCIE for antibodies	Total	Rat liver tissues	Hep-2 cell (preparation 1)	Hep-2 cell (preparation 2)
Ro	**12**	2	8	7
Ro/La	**4**	3	4	4
Ro/La/RNP	**3**	3	3	3
Scl-70	**2**	1	2	1
Total	**21**	**9**	**17**	**15**

Twenty one samples previously shown to contain antibodies to the extractable nuclear antigens (ENA) by the counter current immuno-electrophoresis (CCIE)-method were tested in parallel for antinuclear antibodies (ANA) on Rat liver tissues and two preparations of Hep-2 cell line. Hep-2 - human epithelial cells, Scl-70 - scleroderma-70, RNP - ribonucleic protein

ineffective for the detection of anti-ENA antibodies and the test could potentially miss out 25% of ENA-positive cases. The majority of these cases were positive for anti-Ro-antibodies. Anti-Ro antibodies are the most frequently manifested anti-ENA antibodies in patients with Sjögren's syndrome.[14,18] When the effectiveness of using rat liver tissues for the detection of patients with Sjögren's syndrome was applied, it became apparent that it could potentially miss out approximately 45% of such patients. A number of explanations could account for the lack of sensitivity of the rat liver tissues for the detection of ENA positive samples. Firstly, it has been reported that Ro antigen is either not expressed or found in low amount in rat tissues and this would account for the ANA negative samples that had predominantly anti-Ro antibodies.[9] Secondly, it was also reported that ENA leach out easily from rat liver tissues.[2] Again this would also explain the very few cases with negative ANA, but with positive anti-Ro and anti-La antibodies. Based on the findings of the present study, particularly those regarding antibodies to ENA, it appears that rat liver tissues are ineffective for the detection of CTD. If rat liver tissues are to continue to be used, then a second method of detection for ENA should also be employed in parallel. However, this would be impractical and would lead to a waste of resources and personnel time and a more practical alternative is to switch to the use of Hep-2 cell line. Parallel testing for ANA by rat liver tissues and Hep-2 cell line revealed that the latter tissues are far more sensitive at detecting anti-ENA antibodies and thus as a screening method for Connective Tissue Disease. Moreover, different preparations of Hep-2 cell line had different ability to detect ANA patterns. Therefore, it is important for laboratories considering switching to the Hep-2 cell line to try several preparations before committing themselves to a particular preparation. Hep-2 cells are large dividing cells with large nuclei and nucleoli.[2,9] Therefore, ANA patterns including the nucleoli would be clearer on Hep-2 cells than on rat liver tissues. Moreover, because Hep-2 cells are dividing, the centromere pattern would be present. Furthermore, Hep-2 cells contain large quantity of ENA, including the Ro antigen.[9] Moreover ENA would be less susceptible to leach out from the cells. In addition, other non-nuclear antibodies would be detected, including anti-Jo-1, anti-smooth muscle and anti-mitochondria antibodies, and thus would be used also for the screening of other autoimmune disorders such myositis/dermatomyositis, chronic active hepatitis and primary biliary cirrhosis. It is envisaged that the only draw back with introduction of Hep-2 cell line would be the cost. However, saving in personnel time, cutting rat liver tissues and performing additional assays, combined with the over all benefit it should have on the diagnosis of CTD and other autoimmune diseases, would offset the cost factor.

Finally, since the rate of detection of ANA varies with different Hep-2 cell preparations, laboratories considering switching to these cells should test several preparations before committing themselves to a particular one. Moreover, ANA testing should be run initially on both rat liver tissues and Hep-2 cell line hence, direct comparison can be made and the ANA patterns on Hep-2 cell line are appreciated.

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