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Exposure of HEp-2 Cells to Stress Conditions Influences Antinuclear Antibody Reactivity

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This study of stress-related antinuclear antibody (ANA) reactivity was undertaken with the objective of improving clinical ANA testing. ANA was determined by parallel enzyme-linked immunosorbent assays of crude nuclear protein antigen extracted from HEp-2 cells either grown under optimal conditions (providing nonstress ANA antigen) or exposed to stress (providing stress ANA antigen). The stress stimuli used were gamma radiation (causing DNA damage) and a hypertonic environment (causing apoptosis). Signs of stress-related ANA reactivity were seen among connective tissue disease (CTD) patients (including patients with systemic lupus erythematosus; mixed CTD; calcinosis, Reynaud's phenomenon, esophageal motility disorders, sclerodactyly, and telangiectasia; scleroderma; and Sjögren's syndrome): 11% showed stress-positive ANA (i.e., a significantly stronger ANA reactivity with the extract from stressed cells), whereas 21% showed a markedly weaker reaction with the stress antigen. In contrast, among ANA screening patient sera, with no diagnosis of CTD, the fraction showing stress-positive ANA was higher (7 to 8%, depending on the type of stress) than among those showing a lower reactivity with stress antigen (1.5 to 2.5%). Only one serum among 89 (1%) tested sera from healthy individuals showed a stress-related ANA reaction. This demonstration of stress-related ANA suggests a means to improve the performance of clinical ANA testing.

Antinuclear antibodies (ANA) at high titers and with specific antigen reactivities are typical of systemic autoimmune diseases, including systemic lupus erythematosus (SLE), Sjögren's syndrome, and scleroderma (30, 34). Much interest has been focused on this phenomenon for two major reasons. First, ANA testing is widely used for screening to aid in clinical diagnosis; however, there is a need for performance improvement due to shortcomings in the specificity and sensitivity of ANA as an indicator of systemic autoimmune disease (8, 30, 34). Second, ANA may shed light on basic cellular processes, since it is important for the pathogenesis of this group of diseases (29, 30).

The antigenic targets of ANA show several remarkable characteristics suggestive of a role in the disease mechanism. Although not all of these targets have been identified, they are considered to include only a minority of all nuclear proteins. Furthermore, they are often part of colocalized sets of molecules, such as the spliceosome and the V(D)J recombinase complex (6, 27, 29). A functional denominator for many of these proteins is reactivity with nucleic acids. Since they commonly seem to function in a stress situation, as defined by environmental conditions threatening cellular homeostasis, calling for a recovery process or for apoptosis, ANA have been suggested to indicate an abnormal cellular stress response as a key pathogenesis factor in systemic autoimmune disease (24, 31; Anonymous, Editorial, *Rheumatology* **39**:581–584, 2000). Specifically, this relation to cellular stress has been demonstrated by several reports showing that among ANA targets can be

found (i) DNA repair factors (29), (ii) major heat shock proteins (16, 36), (iii) caspase substrates (4, 5), (iv) phosphorylated nuclear proteins (22, 23, 26, 33), and (v) granzyme B substrates (3).

Thus, many data indicate that proteins being degraded and subsequently expressed on the cell surface (1, 4, 7) during apoptosis are frequent ANA targets. However, ANA are also directed to other nucleic-acid-modifying proteins (e.g., SSA and Sm subcomponents, histones, and Ku86), showing that ANA production is not restricted to apoptosis (3). Instead, experimental data and some hypotheses for the pathogenesis of systemic autoimmune disease fit a more general origin of ANA, including DNA damage, its cellular repair, and the eventual stress situation of apoptosis. Abnormalities in DNA repair have been documented in SLE (2, 12) and Sjögren's syndrome (11, 19), as well as low-rate generation in Sjögren's syndrome patients of chromosome translocations linked to illegitimate V(D)J recombination (13). Hypotheses include those of Harris et al. (12), postulating defective DNA repair as an autoimmunity susceptibility factor, and Fox et al. (9), suggesting an abnormal processing of immunoglobulin and T-cell receptor genes as a basic pathogenetic phenomenon, as well as that of Tak et al. (28), with a scenario of hyperproduction of reactive oxygen species in chronic inflammation, leading to DNA strand breakage, p53 accumulation, and p53 mutation.

In the present work, ANA directed to proteins present specifically in cells exposed to stress conditions has been detected. Many of the DNA repair- and apoptosis-related proteins demonstrated to be widely represented among ANA targets may well also be present in nonstressed cells. Besides a recent demonstration of reactivity of some SLE sera with a stress-modified 70-kDa RNP (10), information on strictly stress-related ANA is, to the best of our knowledge, not yet available.

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Therefore, we have argued that documentation of stress-related ANA would give valuable information in two respects. It would indicate a means to improve the performance of clinical ANA screening, and it would provide direct evidence for a role for cellular stress in the pathogenesis of systemic autoimmune disease.

We have used an enzyme-linked immunosorbent assay (ELISA) protocol with crude nuclear antigen prepared from stressed human Hep-2 cells (i.e., cells committed to apoptosis following exposure to a hypertonic environment or treated with a DNA-damaging agent). The results suggest that stress-related ANA are present in a fraction of patients diagnosed with a connective tissue disease (CTD), such as SLE or Sjögren's syndrome, as well as in sera submitted to a clinical laboratory with a request for ANA screening, but are only rarely present in healthy individuals.

MATERIALS AND METHODS

Sera. Patient sera were from the Lund University Hospital and the serum bank at the Sjögren's Syndrome Research Center, Malmö University Hospital. The screening sera represented consecutive nonselected sera submitted by general practitioners, day care rheumatologists, and hospital wards with a request for ANA testing. After Hep-2 immunofluorescence (IF) ANA testing had been performed in a certified clinical laboratory (Department of Clinical Immunology, Lund University Hospital, using a Hep-2 kit from Euroimmun, Lübeck, Germany), the sera were stored at -20°C until they were used for analysis of stress-specific ANA in the present study. The normal sera came from individuals with suspected gastric ulcer disease and were submitted for determination of antibodies to *Helicobacter pylori*. We assumed these individuals to have a frequency of systemic autoimmune disease not significantly different from that of the general population.

Cell culture. The human epithelial-like tumor line Hep-2 (CCL23) was from the American Type Culture Collection (Manassas, Va.), and the cells were cultured in RPMI 1640 (Gibco, Paisley, United Kingdom) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 25 mM HEPES, and 12 μg of gentamicin/ml at 37°C and 5% CO_2 .

Osmotic induction of apoptosis. Osmotic shock by hypertonic sorbitol treatment was used to induce apoptosis (17). Briefly, subconfluent Hep-2 cultures were incubated with growth medium containing 1 M sorbitol (Sigma, St. Louis, Mo.) at 37°C for 3 h, followed by washing and postincubation in sorbitol-free growth medium at 37°C for up to 3 h before DNA was extracted (nuclear protein was extracted after 3 h).

DNA fragmentation assay. Hep-2 cells were washed with phosphate-buffered saline (PBS) (pH 7.2) and lysed in 30 μl of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% sodium laurylsarcosinate, and 1 mg of proteinase K/ml. The cell lysates were incubated overnight at 50°C , and RNase A (0.3 mg/ml) was then added for another 2 h at 50°C . The lysate was then electrophoresed in a 1.5% agarose gel containing ethidium bromide, and the DNA was visualized under UV radiation (21). A pBR322 DNA molecular weight marker from Roche-Boehringer-Mannheim, Mannheim, Germany (marker X) was used.

Gamma irradiation. Gamma radiation was delivered by a neutron accelerator (Philips, Hamburg, Germany) at a dose rate of 0.70 Gy per min, up to a total dose of 4 Gy, at a distance of 50 cm from the cells (subconfluent cultures) kept in their plastic culture flasks in culture medium at room temperature. The flasks were then returned to the incubator at 37°C and 5% CO_2 . Nuclear protein was extracted 30 min and 1, 2, 8, and 24 h later (one cell flask for each time point); the resulting five extracts were then pooled to generate the stress antigen used for the ANA ELISA analysis. A corresponding nonstress antigen was derived from flasks incubated for 8 h following a mock-irradiation procedure.

Extraction of nuclear protein. The procedure of Schreiber et al. (25) was followed with some modifications. Cytoplasmic protein was removed by lysis of pelleted cells in a neutral pH buffer containing 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 0.5% NP-40, and a mixture of proteinase inhibitors (Complete; Roche). The pelleted cell nuclei were then lysed at 37°C for 3 to 5 min in a hyperosmolar neutral-pH solution with 0.5 M NaCl, 50 mM MgCl_2 , 2 mM CaCl_2 , 10 mM Tris buffer, 100 U of DNase I/ml, and 10% Complete. The protein concentration was determined spectrophotometrically at 540 nm in 96-

well microtiter plates using bicinchoninic acid protein assay reagents (Pierce, Rockford, Ill.). All extracts were stored at -70°C .

ELISA. A conventional microplate immunoassay was performed as described previously, with some modifications (14). Polystyrene microwell plates (F96 Maxisorp; Nunc-Immuno Module, Roskilde, Denmark) were coated with 2 μg of cell nuclear protein per well dissolved in PBS (pH 7.2). The plates were then washed with washing buffer (PBS containing 0.05% Tween 20 [pH 7.4]) and incubated with blocking buffer (PBS containing 1.5% ovalbumin and 0.05% Tween 20 [pH 7.2]). After renewed washing, patient sera diluted 1:3,000 in blocking buffer were added and allowed to react at room temperature for 1 h. The plates were then washed and incubated with peroxidase-conjugated rabbit anti-human immunoglobulin G (IgG; DAKO, Glostrup, Denmark) diluted 1:3,000 in blocking buffer. Finally, the plates were washed again and tetramethylbenzidine in citrate buffer (0.1 M; pH 4.25) with H_2O_2 was added as a substrate. The enzymatic reaction was stopped 10 min later by the addition of 1 M H_2SO_4 , and the optical density (OD) at 450 nm was determined by a spectrophotometer (Multiskan Plus; Labsystems, Espoo, Finland). On each plate was included a calibrator ANA-positive patient serum, which was assigned a fixed OD value of 1.50, permitting comparison of results obtained with different plates and on different dates.

All sera were analyzed with two wells containing stress antigen adjoining two wells with nonstress antigen. Sera showing an OD difference between stress and nonstress antigens of >0.05 were reanalyzed one or two times (with separate microplates and on different dates). Checkerboard titration of antigen concentration versus serum dilution showed coating with 2 μg of nuclear protein per well and 1:3,000 dilution of serum to be optimal (data not shown).

The commercial ANA ELISA kit used (RELISA ANA) was from Immunoconcept (Sacramento, Calif.).

Immunoblotting. The nuclear protein extracts (10 μg) were heated for 10 min at 70°C before being loaded onto a NuPAGE 10% polyacrylamide-Tris-acetate precast gel. The gel was subjected to electrophoresis for 1.5 h at 120 V and then transferred by electroblotting to a polyvinylidene difluoride membrane for 2 h at 25 V. A prestained size marker (NOVEX See Blue prestained standard) was included in each run. After being blocked for 1 h at room temperature with blocking buffer (TBS containing 0.05% Tween 20 and 5% skim milk powder), the membranes were incubated for 1 h at room temperature with patient serum diluted 1:2,000 in blocking buffer. Subsequently, the blots were incubated under gentle agitation at room temperature with a secondary horseradish peroxidase-conjugated antibody diluted 1:4,000 in blocking buffer. The blots were developed using the enhanced chemiluminescence method (Amersham-Pharmacia) with X-ray film or a Bio-Rad Personal Molecular Imager FX.

RESULTS

Performance of in-house ANA ELISA (nonstress antigen).

For the purpose of determining stress-related ANA (i.e., ANA reacting differently with nuclear antigen from stress-treated cells than with antigen from cells cultivated under optimal conditions), we needed to set up an ANA assay serving two basic functions. First, it should truly measure conventional non-stress-related ANA, and second, it must be able to give additional information which could be interpreted to indicate the presence of stress-related ANA. We settled for ELISA, rather than IF, for the main reason that it provides objective data. Nuclear protein was extracted from subconfluent Hep-2 cells and used for a standard microplate ELISA analysis of human serum IgG ANA. A cutoff OD value of 0.45 for the designation of a positive ANA reaction was chosen, based on analysis of 89 normal sera (one serum showed an OD value of 0.45, and a total of four sera reached an OD value of 0.40). Patient sera submitted to a clinical diagnostic laboratory with a request for ANA screening were then analyzed. Comparison with the "gold standard" microscopic Hep-2 IF test showed that our in-house ELISA detected nonstress ANA with acceptable accuracy; the overall agreement with IF was 157 of 200, i.e., very similar to that of a commercially available ANA ELISA kit (156 of 200) (Table 1). However, the sensitivity of

TABLE 1. Specificity and sensitivity of present ANA ELISA compared with a commercial ELISA^a

ANA ELISA and result	HEp-2 IF (gold standard) result	
	No. positive	No. negative
Present		
Positive	10	25
Negative	18	147
Commercial		
Positive	24	37
Negative	7	132

^a Separate groups of ANA-screened patient sera were used for the two ANA ELISA methods ($n = 200$ for each group).

our ELISA was lower than that of the commercial ELISA (10 out of 28 IF-positive sera were scored as positive, compared with 24 out of 31 for the commercial ELISA). This may be explained partly by different nuclear protein extraction procedures; ours was designed primarily for isolation of non-DNA-linked elements (assumed to include most nuclear stress-related proteins) rather than chromatin. This assumption is supported by the observation that only 3 out of the 14 ANA IF-positive sera with the homogeneous IF pattern gave a positive reaction in our ANA ELISA, while 6 of the 8 ANA IF speckled-pattern sera were positive in our ANA ELISA. The IF titers for the discrepant sera that were IF positive but lacked ANA ELISA reactivity were often low: 10 of the homogeneous IF pattern sera were low titer and were assigned a value of 14 IU, and 1 had a higher titer corresponding to 54 IU; the 2 speckled-pattern IF sera showed titers corresponding to 14 and 217 IU, respectively.

Stress-related ANA reactions. Due to the reported frequent involvement of apoptosis-related proteins in ANA formation, we defined stress conditions leading to apoptosis. HEP-2 cells were treated with a hypertonic sorbitol solution and then incubated under optimal growth conditions, permitting the cells to develop apoptosis characteristics (17). DNA was extracted, size separated on an agarose gel, and visualized by ethidium bromide staining. The hypertonic treatment per se was found to induce some DNA fragmentation (Fig. 1). However, within 15 min of incubation in sorbitol-free growth medium, a fragmentation pattern compatible with internucleosomal degradation became evident. This sign of apoptosis appeared to reach a maximum at 60 min of incubation. This time-dependent development of DNA fragmentation indicates that apoptosis-specific stress changes were induced in at least a fraction of the cells in response to the osmotic stress stimulus (Fig. 1).

ELISA microplate wells were coated with nuclear protein extracted from HEP-2 cells grown under optimal conditions and after a sorbitol stress protocol yielding an apoptosis reaction. The binding of ANA screening patient sera and of normal sera to these two extracts of IgG was then analyzed. Among the 89 normal sera, there was some difference in the results obtained using adjoining ELISA microplate wells coated with nonstress and stress antigens, respectively. Twenty-eight sera gave a higher OD value with stress antigen (the largest difference was 0.04, except for a single serum showing OD values of 0.54 for stress antigen and 0.36 for nonstress antigen), whereas 14 sera presented a higher OD value with nonstress antigen (the largest difference was 0.03) and the remaining 47 sera

showed no difference (i.e., ODs of <0.01). All these differences (except for the stress-related OD of 0.18 [0.54 – 0.36] in the single reactive serum) were considered nonsignificant and due to a variation inherent in the method. The standard deviation (SD) among the OD values of these 89 sera was 0.02, reflecting the sum of the method variation caused by technical factors plus possibly a low-grade stress-specific ANA reactivity in some sera. We also used results obtained with a serum showing a strongly positive IF ANA titer to get additional information on the method variation, guiding us to consider a stress-specific OD reaction in a patient serum to be a true marker of stress-related ANA rather than the result of technical method variation. This serum was analyzed on each plate to serve as a calibrator (with an assigned OD value of 1.50) and showed a variability larger than that seen in the low-titer control sera; the SD for the stress-related OD difference was 0.07. Since the large majority of our tested patient sera produced OD values in a lower range than this strong calibrator, we were guided by the variation among the normal sera when defining our criteria for a stress-related ANA reaction: a difference in OD of >0.05 between the stress and nonstress antigens, corresponding to >2.5 SD of the same difference seen among the normal sera. To further reduce the influence of chance on the results, for a serum to be assigned a stress-related ANA reactivity, this difference should be obtained in each of two or three independent assays. Using these stringent criteria, the chance of falsely designating a patient serum as containing stress-related ANA should be less than 1 in 100.

Among the consecutive series of 200 ANA screening patient sera assayed with antigen from hypertonic-treatment HEP-2 cells, 17 (8.5%) were considered to show stress-positive ANA (i.e., a significantly stronger ANA reactivity was seen with the extract from stressed cells than with that from nonstressed

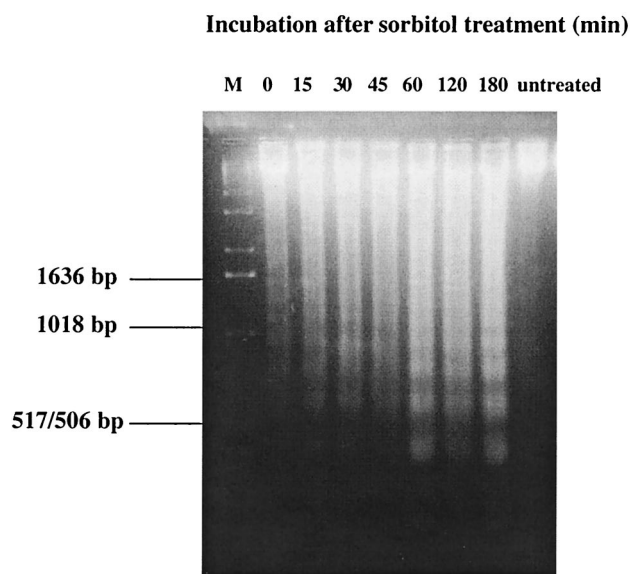


FIG. 1. DNA fragmentation indicating apoptosis in stress-treated HEP-2 cells. Subconfluent HEP-2 cells were exposed to a hypertonic 1 M sorbitol solution for 3 h, followed by washing and further incubation in growth medium at 37°C. Cellular DNA was extracted at the indicated times and analyzed by agarose gel electrophoresis and ethidium bromide staining. M, DNA molecular size standard (in base pairs).

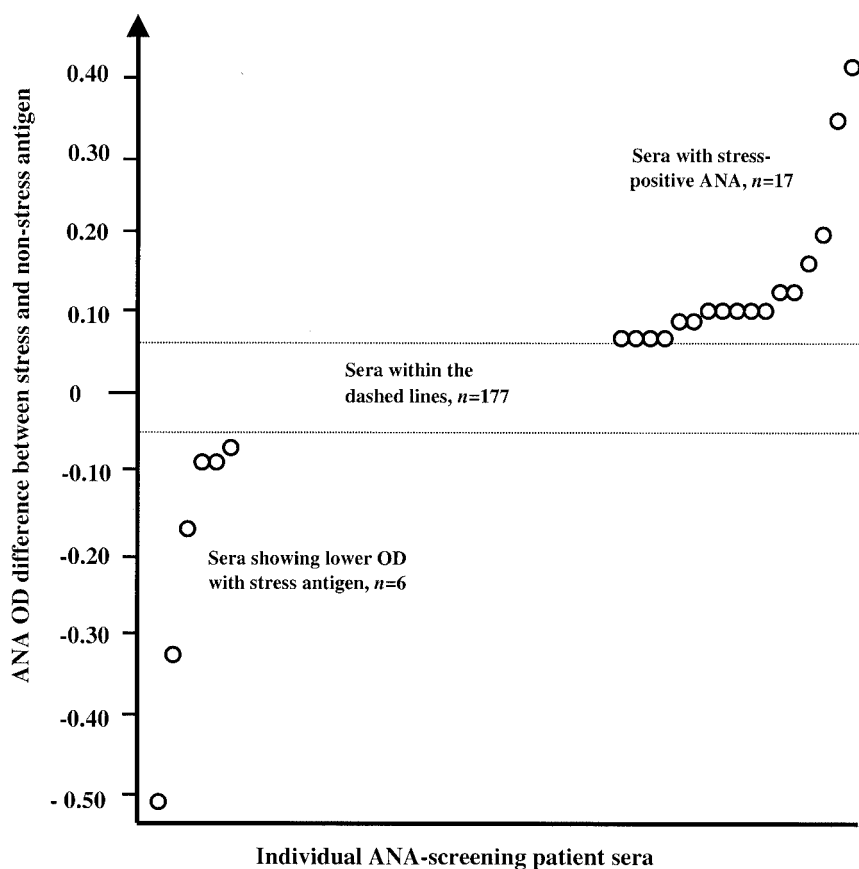


FIG. 2. Stress-specific ANA in a series of 200 consecutive ANA screening patient sera. The binding of IgG to nuclear protein extracted from HEp-2 cells either grown under ideal conditions or stressed with a hypertonic sorbitol solution was determined by two parallel ELISAs. Each circle represents one serum and shows the difference in OD between the stress antigen and the nonstress antigen. The sera are arranged according to the size of this difference. The 177 sera showing no or a small OD difference (i.e., within the two dotted lines) are not designated with circles. The cutoff OD level of 0.05 (indicated by the two dotted lines) was used for designation of a stress-specific ANA reaction.

cells), and a smaller fraction (6 sera [3%]) showed a weaker reaction with stressed wells (i.e., the OD value for stress antigen was >0.05 lower than that for the nonstress antigen) (Fig. 2). Three out of these six “stress-negative” sera were located close to the OD cutoff of 0.05 (and may possibly be indicative of the size of the method variation), whereas the stronger reaction for the remaining three sera (OD values of 0.17, 0.34, and 0.51) may reflect a decrease in some antigenic components occurring during the cellular stress situation (Fig. 2). Among the larger group of 17 stress-positive sera, 6 showed relatively strong reactions (ODs of >0.10), most unlikely explained by method variation, while some of the results for the 11 sera with stress-related reactions with ODs of 0.05 to 0.10 (although determined to be stress positive in at least two independent experiments) possibly can be attributed to a random method variation event. The full ANA results for these 17 stress-positive sera are presented in Fig. 3. The strengths (ODs) of the stress-positive ANA reactions ranged from 0.06 to 0.40. Interestingly, 6 of these 17 sera were found to be negative for non-stress-related conventional ANA when analyzed with our in-house ELISA.

The stress-related ANA reactivity is not limited to the hypertonic-treatment antigen, since we obtained a similar result with another group of ANA screening patient sera tested with

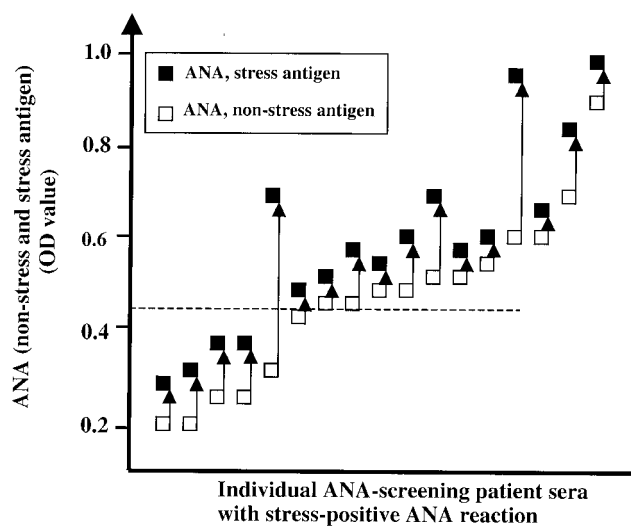


FIG. 3. ANA results for the 17 ANA screening patient sera (same individuals shown in Fig. 2) showing stress-positive ANA activity with the hypertonic-treatment stress antigen. The arrows point to the higher OD values obtained using stress antigen (solid symbols) compared with those obtained with nonstress antigen (open symbols). The dashed line indicates the cutoff OD value for designation of a positive reaction in conventional ANA using nonstress antigen.

TABLE 2. ANA ELISA reactivities in different patient groups with stressed HEP-2 cells

Patient group	Total no. of patients	Reactivity ^a			
		Stress-positive patients		Stress-negative patients	
		<i>n</i> (%)	IF ANA negative	<i>n</i> (%)	IF ANA negative
ANA-screened population					
Hypertonic sorbitol treatment antigen	194	16 (8)	12	3 (1.5)	2
Gamma irradiation antigen	194	14 (7)	11	5 (2.5)	2
CTD patients					
SLE or MCTD	12	2 (17)	0	5 (42)	0
Scleroderma	6	0 (0)	0	1 (17)	0
Sjögren, selected for SSA ⁺ -SSB ⁺	20	2 (10)	NA	2 (10)	NA
Total	38	4 (11)	NA	8 (21)	NA
Rheumatoid arthritis	20	1 (5)	1	0	0
Control subjects	89	1 (1)	1	0	0

^a Sera showing highest ODs with nuclear antigen from stressed cells are designated stress positive, and those sera showing lowest ODs with nuclear antigen from stressed cells are designated stress negative. Hypertonic sorbitol treatment antigen was used for all sera except the indicated gamma-irradiated ANA screening group. NA, not applicable.

antigen from HEP-2 cells exposed to gamma radiation. This DNA-damaging agent was chosen due to the frequent representation of DNA repair factors among ANA targets. The gamma radiation stress antigen was not analyzed with normal sera, thus providing no clear guidance for the selection of a cutoff OD value for designation of a stress-related ANA reaction. Therefore, an arbitrary ELISA cutoff value for stress-related ANA (i.e., for the difference in OD between the two types of antigen) was set at an OD of 0.10. Among this group of 200 ANA screening patient sera, 16 (8%) were then found to contain stress-positive ANA with an OD range of 0.10 to 0.60. As with the analysis of hypertonic-treatment HEP-2 antigen, a smaller fraction of the sera showed a weaker reaction with the stress antigen; six (3%) sera showed corresponding OD values of <0.10 (range, -0.10 to -0.35).

For most of the ANA screening patients, no clinical data were provided by the requesting physician. However, some patients were diagnosed with a specific CTD: within the hypertonic-treatment antigen group, there were two patients with SLE, two patients with SLE or MCTD, one patient with Sjögren's syndrome, and one patient with calcinosis, Reynaud's phenomenon, esophageal motility disorders, sclerodactyly, and telangiectasia (CREST); within the gamma radiation group, there were one patient with SLE, three patients with Sjögren's syndrome, and two CREST patients. In order to compare the results for nondiagnosed screened patients with those for CTD patients, the CTD patients were excluded from the screened populations and grouped together with sera obtained from additional CTD patients; the data resulting after this regrouping are shown in Table 2. The two screening populations gave very similar results, with a larger fraction (7 to 8%) showing a stress-positive reaction compared with 1.5 to 2.5% for the nonstress antigen. In general, a stronger reactivity was observed among the CTD patients, with 11% of the sera showing a stronger reaction with stress antigen. However, in contrast to the screened cases, CTD patients presenting a weaker reaction with stress antigen were markedly more frequent (21%). This was especially noted for SLE (42%). In the Sjögren's syndrome group, two patients (10%) showed enhanced as well as reduced

reactivity with stress antigen. A total of 27 IF ANA-negative sera in the two screened populations showed stress-related ANA reactivity, indicating that the sensitivity of ANA testing may be improved by the use of stress antigen. In contrast to the ANA screening sera, all of the CTD sera with stress-related reactivity (*n* = 12) were ANA IF positive (Table 2). It should be noted, though, that the majority of the CTD patients (i.e., the Sjögren's syndrome sera [*n* = 20]) were selected for IF ANA and SSA-SSB positivity. Therefore, our data cannot exclude the possibility that stress-related ANA activity is also present among ANA IF-negative CTD patients.

Immunoblotting confirmation of stress-related ANA ELISA reactivity. In order to further document the specificity of our findings of stress-related ANA ELISA activity, immunoblotting was performed using nuclear protein that had been size separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and a total of 35 patient sera. Agreement between the two immunoassays was anticipated, although discordant results may occur due to the stronger protein denaturation employed in the electrophoresis method. Ten sera with stress-positive ANA-ELISA results were included; four showed bands with markedly stronger intensity using stress antigen, compared with the neighboring gel lane containing nonstress antigen (illustrated by serum 4 [Fig. 4]), one showed equal banding patterns, one serum was without bands, and for four sera no information was obtained due to a high nonspecific background binding to the membrane. Immunoblotting was also done with three sera having stress-negative ANA ELISA results; two sera presented weaker bands with the stress antigen (sera 1 and 3 [Fig. 4]), whereas the third serum showed no band differences between the two antigen types. Thus, for the majority (six of nine) of the informative sera, the result of stress-related ANA ELISA reactivity could be confirmed by Western blotting. For comparison, the ODs from ANA ELISA for the respective sera are indicated at the bottom of Fig. 4. There was a large variation in the apparent molecular masses of the proteins detected for both stress-positive and stress-negative reactivities, although some bands could be seen with more than one serum, e.g., the approximately 150-kDa stress-

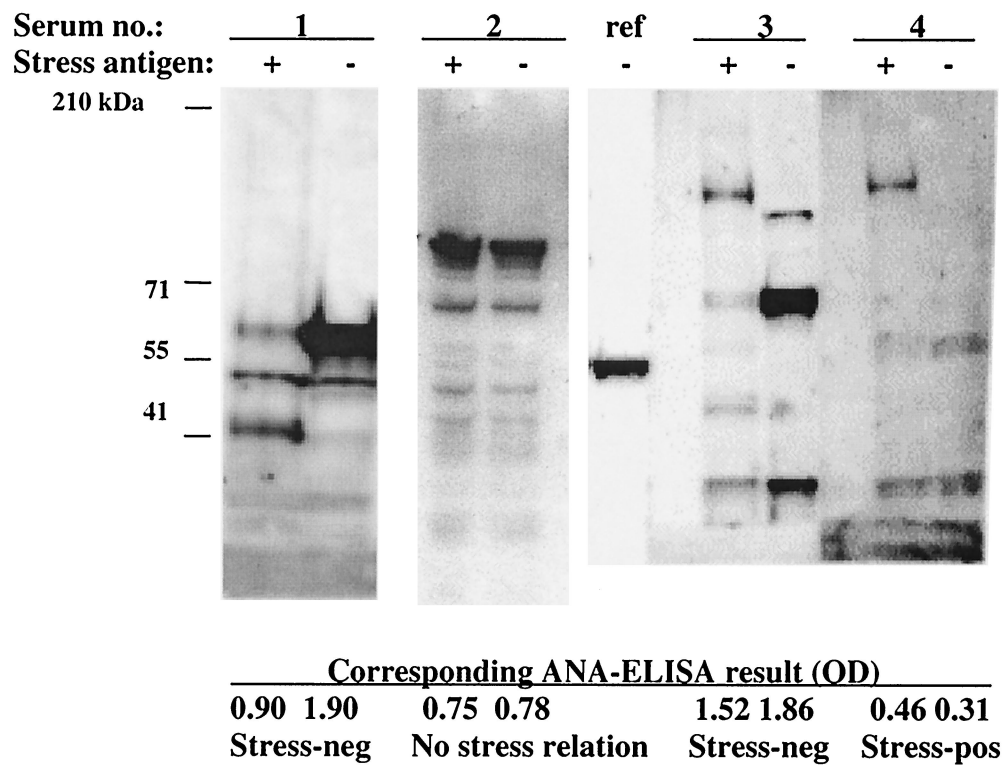


FIG. 4. Reactivity in immunoblotting of sera showing a stress-related ANA ELISA result. ref, reference serum obtained from an SSA-SSB-positive Sjögren's syndrome patient. The type of stress antigen used is indicated as follows: +, from HEP-2 cells exposed to a stress hypertonic sorbitol treatment; -, from nonstressed cells. For comparison, the OD values resulting from ANA ELISA are included.

positive band for sera 3 and 4 (Fig. 4). None of the additional 22 sera analyzed by immunoblotting, all lacking stress-related ANA ELISA reactivity, showed any evidence of banding differences due to the antigen stress condition (exemplified by serum 2 [Fig. 4]). It is interesting that for some sera the immunoblotting revealed signs of both stress-positive and stress-negative reactivities. This is clearly demonstrated by serum 1 (Fig. 4), with an approximately 40-kDa band only in the stress-positive lane but with its largest total band intensity in the stress-negative lane due to a heavy approximately 60-kDa band; i.e., for this serum, the immunoblotting result was consistent with that of the stress-negative ANA ELISA.

DISCUSSION

It may be taken as an argument against the objective of the present study that ANA reacting with stress-related proteins is a well-known phenomenon. Indeed, during the 1980s there were many reports showing an immune response to heat shock proteins in patients with systemic autoimmune disease and other sources of chronic inflammation (16, 30, 36), although ANA to heat shock proteins were noted to be infrequent (16). This immunoreactivity was thought to result from molecular mimicry, i.e., from a defense reaction against prokaryotic molecules having much homology with human cellular components; the basic pathogenetic mechanism was then thought to be cross-reactivity in the patients' immune systems. Later, starting with the discovery of deficiency of Fas protein and apoptosis in the SLE model *lpr* mouse strain (35), more atten-

tion was focused on a possible etiological role of a primary abnormality in the cellular stress response of systemic autoimmune disease patients (31). In support of this idea, it has recently been clearly shown that apoptosis-related proteins are frequent targets of ANA (3, 4, 5, 22, 23, 24, 26, 33). A possible mechanism for the immunogenicity of these proteins has also been presented, i.e., the exposure of nuclear protein on the apoptotic cell surface (1, 4, 7).

However, ANA formation cannot be linked fully to apoptosis, since a number of frequent ANA targets (e.g., SSA, Sm, and Ku86) are not cleaved by caspases or granzyme B (3). Apoptosis can be viewed as the ultimate cellular stress response chosen by severely damaged cells, whereas a milder form of stress is met by the cell with a recovery attempt, including DNA repair. An abnormality in such a recovery process is suggested by reports of DNA repair alterations in SLE and Sjögren's syndrome patients (2, 11, 12, 19) and by our observation of an enhanced cell cycle arrest in gamma-irradiated Sjögren's syndrome lymphocytes (G. Henriksson et al., submitted for publication). An interesting report of the SSB autoantigen showing promoter gene switching and alternative splicing specific for a Sjögren's syndrome patient also indicates a primary defect in the antigenic targets of ANA (32). A stress response not leading to apoptosis can be assumed to be potentially immunogenic, considering the general model for antigenic recognition proposed by Matzinger (20), based on sensing by the lymphocytes of danger rather than nonself structures.

The present demonstration of stress-related ANA extends

the available data on ANA and cellular stress. The enhanced reactivity of some patient sera with factors that are upregulated in stressed cells provides evidence for a role for cellular stress in ANA formation. In some other sera, the opposite kind of stress-related ANA ELISA activity was seen, i.e., a lower reactivity with antigen from stressed cells than with nonstress antigen. This stress-negative result may reflect a reduction in concentration of ANA binding to cellular components during a cellular stress response. A number of alterations can be envisaged to occur during stress in the epitopes recognized by patient ANA. During a recovery phase characterized by repair processes, the synthesis of several proteins is induced (15), while some factors needed for proliferation are probably reduced in quantity. Similarly, in severely damaged cells going into apoptosis, protein cleavage by caspases and granzyme B can be assumed to generate new epitopes as well as to eliminate native protein configurations (4, 5). In addition, the binding of phosphorylation-specific ANA will be affected by kinases and phosphatases acting during a stress response. The stress conditions employed by us (hypertonic treatment and gamma irradiation) can be assumed to generate a spectrum of stress responses governed by, e.g., cell cycle phase distribution and leading to the inclusion in our nuclear extracts of some of the protein alterations reported to occur during cellular recovery and apoptosis (18).

It is tempting to speculate that the relatively high prevalence of stress-positive ANA in ANA screening patients (7% presented a higher OD and 1.5 to 2.5% presented a lower OD with stress antigen) reflects reactivity with inducible recovery factors, whereas the dominant result for stress-negative reactivity among the CTD patients (11% reacted more strongly with stress antigen, whereas 21% showed weaker reactivity) reflects a reduction in the amount of antigenicity of cellular proteins during apoptosis (leading to a lower ANA OD result with stress antigen). Interestingly, our observations with screened patients may indicate that a specific patient group (separate from the disease entities conventionally included in CTD) is located within the ANA-screened non-CTD population.

In summary, the frequency of stress-related ANA in ANA-screened patients being negative in conventional ANA testing suggests a potential for improvement of the current ANA-screening procedure. However, for the diagnostic value to be assessed, the clinical characteristics of stress-positive ANA-screened patients must be determined. Further work is also needed to identify the target protein reactive with stress-related ANA, as well as to define the stress conditions and the detection system best suited to clinically useful stress antigen ANA testing.

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REFERENCES

- Bachmann, M., S. Chang, A. Bernd, W. Mayet, K. H. Meyer zum Büschenfelde, and W. E. Müller. 1991. Translocation of the nuclear autoantigen La to cell surface: assembly and disassembly with the extracellular matrix. *Autoimmunity* 9:99-107.
- Bashir, S., G. Harris, M. A. Denman, D. R. Blake, and P. G. Winyard. 1993. Oxidative DNA damage and cellular sensitivity to oxidative stress in human autoimmune diseases. *Ann. Rheum. Dis.* 52:659-666.
- Casciola-Rosen, L., F. Andrade, D. Ulanet, W. B. Wong, and A. Rosen. 1999. Cleavage by granzyme B is strongly predictive of autoantigen status: implications for initiation of autoimmunity. *J. Exp. Med.* 190:815-825.
- Casciola-Rosen, L. A., G. Anhalt, and A. Rosen. 1994. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J. Exp. Med.* 179:1317-1330.
- Casiano, C. A., and E. M. Tan. 1996. Antinuclear autoantibodies: probes for defining proteolytic events associated with apoptosis. *Mol. Biol. Rep.* 23:211-216.
- Cooley, H. M., B. J. Melny, R. Gleeson, T. Greco, and T. W. Kay. 1999. Clinical and serological associations of anti-Ku antibody. *J. Rheumatol.* 26:563-567.
- Dörner, T., M. Hucko, W. J. Mayet, U. Trefzer, G. R. Burmester, and F. Hiepe. 1995. Enhanced membrane expression of the 52 kDa Ro(SS-A) and La(SS-B) antigens by human keratinocytes induced by TNF- α . *Ann. Rheum. Dis.* 54:904-909.
- Emlen, W., and L. O'Neill. 1997. Clinical significance of antinuclear antibodies: comparison of detection with immunofluorescence and enzyme-linked immunosorbent assays. *Arthritis Rheum.* 40:1612-1618.
- Fox, R. I., S. Fong, T. P. Chen, and T. J. Kipps. 1988. Autoantibody production in Sjögren's syndrome: a hypothesis regarding defects in somatic diversification of germ line encoded genes. *In Vivo* 2:47-56.
- Greidinger, E. L., L. Casciola-Rosen, S. M. Morris, R. W. Hoffman, and A. Rosen. 2000. Autoantibody recognition of distinctly modified forms of the U1-70-kd antigen is associated with different clinical disease manifestations. *Arthritis Rheum.* 43:881-888.
- Guo, K., G. Major, H. Foster, M. Bassendine, J. Collier, D. Ross, and I. Griffiths. 1995. Defective repair of O⁶-methylguanine-DNA in primary Sjögren's syndrome patients predisposed to lymphoma. *Ann. Rheum. Dis.* 54:229-232.
- Harris, G., L. Asbery, P. D. Lawley, A. M. Denman, and W. Hylton. 1982. Defective repair of O⁶-methylguanine in autoimmune diseases. *Lancet* ii:952-956.
- Henriksson, G., M. Brant, Z. Sandor, R. Manthorpe, and A. Bredberg. 1999. Sjögren's syndrome: lymphoma predisposition coupled with a reduced frequency of t(14;18) translocations in blood lymphocytes. *Mol. Carcinog.* 24:226-231.
- Henriksson, G., R. Manthorpe, and A. Bredberg. 2000. Antibodies to CD4 in primary Sjögren's syndrome. *Rheumatology* 39:142-147.
- Holbrook, N. J., and A. J. Fornace. 1991. Response to adversity: molecular control of gene activation following genotoxic stress. *New Biol.* 3:825-833.
- Jarjour, W. N., B. D. Jeffries, J. S. Davis, W. J. Welch, T. Mimura, and J. B. Winfield. 1991. Autoantibodies to human stress proteins. *Arthritis Rheum.* 34:1133-1137.
- Koyama, A. H., and Y. Miwa. 1997. Suppression of apoptotic DNA fragmentation in herpes simplex virus type 1-infected cells. *J. Virol.* 71:2567-2571.
- Kyriakis, J. M., and J. Avruch. 1996. Sounding the alarm: protein kinase cascades activated by stress and inflammation. *J. Biol. Chem.* 271:24313-24316.
- Lavasani, S., G. Henriksson, M. Brant, A. Henriksson, M. Radulic, R. Manthorpe, and A. Bredberg. 1998. Abnormal DNA damage-inducible protein in cells from Sjögren's syndrome patients. *J. Autoimmun.* 11:363-369.
- Matzinger, P. 1994. Tolerance, danger, and the extended family. *Annu. Rev. Immunol.* 12:991-1045.
- Mondino, A., and M. K. Jenkins. 1995. Accumulation of sequence-specific RNA-binding proteins in the cytosol of activated T cells undergoing RNA degradation and apoptosis. *J. Biol. Chem.* 270:26593-26601.
- Neugebauer, K. M., J. T. Merrill, M. H. Wener, R. G. Lahita, and M. B. Roth. 2000. SR proteins are autoantigens in patients with systemic lupus erythematosus: importance of phosphoepitopes. *Arthritis Rheum.* 43:1768-1778.
- Overzet, K., T. J. Gensler, S. J. Kim, M. E. Geiger, W. J. van Venrooij, K. M. Pollard, P. Anderson, and P. J. Utz. 2000. Small nucleolar RNP scleroderma autoantigens associate with phosphorylated serine/arginine splicing factors during apoptosis. *Arthritis Rheum.* 43:1327-1336.
- Rosen, A., and L. Casciola-Rosen. 1999. Autoantigens as substrates for apoptotic proteases: implications for the pathogenesis of systemic autoimmune disease. *Cell Death Differ.* 6:6-12.
- Schreiber, E., P. Matthias, M. M. Müller, and W. Schaffner. 1989. Rapid detection of octamer binding proteins with 'mini-extracts' prepared from a small number of cells. *Nucleic Acids Res.* 17:6419.
- Stetler, D. A., and S. T. Jacob. 1984. Phosphorylation of RNA polymerase I augments its interaction with autoantibodies of systemic lupus erythematosus patients. *J. Biol. Chem.* 259:13629-13632.
- Suwa, A., M. Hirakata, Y. Takeda, Y. Okano, T. Mimori, S. Inada, F. Watanabe, H. Teraoka, W. S. Dynan, and J. A. Hardin. 1996. Autoantibodies to DNA-dependent protein kinase. Probes for the catalytic subunit. *J. Clin. Invest.* 97:1417-1421.
- Tak, P. P., N. J. Zvaifler, D. R. Green, and G. S. Firestein. 2000. Rheumatoid arthritis and p53: how oxidative stress might alter the course of inflammatory diseases. *Immunol. Today* 21:78-82.
- Tan, E. M. 1989. Interactions between autoimmunity and molecular and cell biology: bridges between clinical and basic sciences. *J. Clin. Invest.* 84:1-6.

30. **Tan, E. M.** 1989. Antinuclear antibodies: diagnostic markers for autoimmune diseases and probes for cell biology. *Adv. Immunol.* **44**:93–151.
31. **Tan, E. M.** 1997. Autoantibodies and autoimmunity: a three-decade perspective. *Ann. N. Y. Acad. Sci.* **815**:1–13.
32. **Troster, H., T. E. Metzger, L. Semsei, M. Schwemmle, A. Winterpacht, B. Zabel, and M. Bachmann.** 1994. One gene, two transcripts: isolation of an alternative transcript encoding for the autoantigen La/SS-B from a cDNA library of a patient with primary Sjögren's syndrome. *J. Exp. Med.* **180**:2059–2067.
33. **Utz, P. J., M. Hottelet, P. H. Schur, and P. Anderson.** 1997. Proteins phosphorylated during stress-induced apoptosis are common targets for autoantibody production in patients with systemic lupus erythematosus. *J. Exp. Med.* **185**:843–854.
34. **von Mühlen, C. A., and E. M. Tan.** 1995. Autoantibodies in the diagnosis of systemic rheumatic diseases. *Semin. Arthritis Rheum.* **24**:323–358.
35. **Watanabe-Fukunaga, R., C. I. Brannan, N. G. Copeland, N. A. Jenkins, and S. Nagata.** 1992. Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis. *Nature* **356**:314–317.
36. **Winfield, J. B., and W. N. Jarjour.** 1991. Stress proteins, autoimmunity, and autoimmune disease. *Curr. Top. Microbiol. Immunol.* **167**:161–189.