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Antinucleolar Antibodies and Their Disease Association

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SUMMARY **The prevalence of the antinucleolar antibodies (ANoA) demonstrated by indirect immunofluorescence technique in 1,662 sera of patients with a known or suspected rheumatic disease increased from 1.97% when mouse kidney (MK) was used as substrate to 4.9% when HEp-2 cells were used as substrate. However, an appropriate commercial HEp-2 substrate must be selected in order to increase the sensitivity of ANoA positivity. There were 3 distinct staining patterns of the nucleolar immunofluorescence: homogeneous speckle, and clumpy. Irrespective of the patterns, the most common diagnoses among patients who had ANoA were systemic sclerosis (PSS) and systemic lupus erythematosus (SLE); 36% and 35%, respectivelt). On the contrary, the incidence of these antibodies in PSS was 41% while it was ony 3% in SLE patients. Almost all patients with speckled nucleolar staining had PSS as their underlying disease while most of the patients with homogeneous nucleolar staining had SLE. No distinct correlation between the different nucleolar staining patterns and specific organ involvements in our lupus and PSS patients was found except for the higher frequency of clumpy staining in male scleroderma with no joint involvement.**

This study demonstrates that: 1) ANoA are uncommon in unselected sera although use of a cell line substrate doubles the rate of positivity; 2) the proper HEp-2 substrate is critical in the detection of ANoA; 3) PSS and SLE are the most frequent diseases associated with ANoA but the frequency of these antibodies in SLE patients was very low. ; 4) there are 3 distinct nucleolar staining patterns which may be associated with different rheumatic diseases; and 5) compared with ANoA negative scleroderma, clumpy nucleolar staining had significantly higher incidence in men with no joint involvement but a tendency towards more lung manifestatons.

The presence of antinucleolar antibodies (ANoA) in the sera of patients with systemic rheumatic disease was first described by Beck in 1961.[1] They are usually found in the sera of patients with progressive systemic sclerosis (PSS). The frequency of ANoA positivity by the indirect immunofluorescence technique in various systemic rheumatic diseases had been reported,[2-4] but those were limited to the use of rat liver or mouse kidney as substrate. Utilising tissue culture cells as substrate, ANoA were detected in a higher frequency in PSS[5] and could be easily separated into several patterns. However, a comparison of the sensitivities among various substrates in detection of ANoA has not been defined. Moreover, correlations between different ANoA and various connective tissue diseases have thus far not been established.

The purpose of our study was to determine the sensitivity of different substrates for ANoA positivity and to determine whether there was any disease or clinical association with the different nucleolar staining patterns. As the classification of nucleolar morphologies can be easily done by the indirect immunofluorescence technique, it may be a useful tool for confirming diagnosis and predicting prognosis of certain autoimmune diseases.

MATERIALS AND METHODS

Substrates

We used 10 different kinds of

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substrates which could be separated into two groups:

1. Tissue sections consisted of mouse kidney (MK), mouse liver (ML), rat kidney (RK) and rat liver (RL). Each of them was snap frozen in liquid nitrogen, embedded in optimal control temperature fluid (OTC), cut into 4 μm thickness by a cryostat and mounted on glass slides. The sections were fixed in acetone for 10 minutes at room temperature and air dried just prior to use.

2. Tissue cultures consisted of 5 different commercially available HEp-2 cells from Antibodies Inc. (Davis, California), Kallestad Diagnostics (South Austin, Texas), Bion (Park Ridge, IL), Immunoconcepts Inc. (Sacramento, Ca) and Meloy Laboratories (Springfield, Va). Apart from these cell lines from human laryngeal carcinoma, HEp-2, we also prepared our own KB cells for comparison. These adherent cells, originally propagated from a human floor of mouth carcinoma, were maintained in TC-flask (Nunc) with 10 Eagle minimum essential medium. After 72 hours in a CO_2 incubator, the monolayer cells were washed by Hank's balanced salt solution and were detached by trypsin treatment. With added medium, cells were recultured on glass slide within the moist chamber and were reincubated in a CO_2 incubator for another 24 hours before being washed by PBS and fixed in acetone for 10 minutes at room temperature.

Study protocol

Sera from 1,622 consecutive patients with suspected or known rheumatic disease sent to the rheumatology laboratory at Ramathibodi Hospital during a one year period were tested for the presence of the antinucleolar antibodies (ANoA) by a standard indirect immunofluorescence technique[7] on two different substrates. One consisted of the MK sections routinely used in our laboratory. The other was the HEp-2 cells from the same company (Antibodies Inc.) as previously used by Bernstein *et al*[6] for morphological classification of ANoA.

Sera were diluted in phosphate buffered saline, pH 7.4 at 1:8 dilution for MK substrate and 1:40 dilution for HEp-2 cells. A polyspecific sheep antiserum to human immunoglobulin conjugated with fluorescein isothiocyanate (Welcome Reagents Limited, London, England) was used as the detecting reagent. In the case of tissue culture substrates, the preparations were counterstained for 30 seconds with 0.05% Evans blue in 0.01 M phosphate buffer, 0.15 M sodium chloride, pH 7.3. The intensity of fluorescent nucleolar staining was graded from 0-4+ on an Olympus fluorescence microscope. Sera giving 1+ or greater fluorescent nucleolar pattern were considered positive.

The nucleolar staining patterns were further classified according to a system described by Bernstein *et al*[6] on the Antibodies Inc. HEp-2 slides. The serum specimens were tested for nucleolar staining morphology without the tester having any knowledge of the clinical situation. Subsequently, the clinical diagnoses and organ manifestations were compared with the different nucleolar staining patterns. Diagnoses were made using the American Rheumatism Association criteria[8] or classic clinical and laboratory findings. Student *t* test, Fischer's exact test and Chi square methods were used, where appropriate, to determine the statistical significance ($p \leq 0.05$) of observed differences.

Sera with ANoA were next examined on several different substrates as described above (ie ML, RK, RL, KB cells, and HEp-2 slides from Kallestad, Bion, Immunoconcepts and Meloy) in order to determine the sensitivity of each substrate for ANoA test. Each positive sera were tested at 1:8 dilution and 1:40 dilution for tissue section substrates and tissue culture substrates, respectively.

Another 100 normal human sera were also examined on the HEp-2 slides from Antibodies Inc. for the presence of ANoA.

RESULTS

Incidence

The sera of 32/1,622 patients (1.97%) demonstrated ANoA on MK substrates while 81/1,622 sera (4.9%) had ANoA when HEp-2 cells were used as substrates. Nucleolar staining was not produced by the healthy control sera. The distribution of ANoA according to diagnosis indicates that these autoantibodies are more commonly found in PSS (29/81 or 36%) and systemic lupus erythematosus (SLE; 28/81 or 35%) than in the other rheumatic disorders (Table 2). The rest of the patients with ANoA had rheumatoid arthritis (6), Raynaud's disease (4), polydermatomyositis (3), juvenile

Table 1. Incidence of the ANoA

Sera	ANoA positivity (%)	
	Mouse kidney	HEp-2 cells
Rheumatic disease (n=1,622)	32 (1.97)	81 (4.9)
Healthy control (n=100)	0	0

Table 2. Frequency and profiles of ANoA within each disease category

Diagnoses	No. of patients diagnosed	No. of patients with ANoA (%)†	HEp-2 nucleolar pattern		
			Homo* (n=48)	Clumpy (n=18)	Speckle (n=15)
Systemic scleroderma	71	29 (41)	7	8	14
Lupus erythematosus	802	28 (3)	24	3	1
Rheumatoid arthritis	97	6 (6)	4	2	-
Raynaud's disease	10	4 (40)	3	1	-
Polymyositis	21	3 (14)	3	-	-
Drug induced ANA	15	2 (13)	1	-	-
Juvenile arthritis	19	2 (11)	1	1	-
Discoid lupus	38	2 (5)	2	-	-
Primary Sjogren's	10	1 (10)	1	-	-
Reiter's syndrome	29	1 (3)	1	-	-
Polymyalgia rheumatica	11	1 (9)	1	-	-
Hashimoto's thyroiditis	5	1 (20)	-	1	-
Interstitial lung fibrosis	6	1 (17)	-	1	-

*Homo = homogeneous nucleolar patterns

†Percent within each disease category.

arthritis (2), discoid lupus (2), hydralazine induced antinuclear antibodies (2), primary Sjogren's syndrome (1), Reiter's syndrome (1), polymyalgia rheumatica (1), Hashimoto's thyroiditis (1) and occupational related interstitial pulmonary fibrosis (1).

From the large pool (1,622) of patients, we extended our study to look for the distribution of incidence of each disease which showed ANoA. Frequency of ANoA within each disease category was demonstrated in Table 2. These antibodies were more commonly found among patients with scleroderma (41%) and Raynaud's disease (40%). Their incidences in Hashimoto's thyroiditis, interstitial lung fibrosis and polymyositis were 20%, 17% and 14%, respectively. The incidence was lowest among SLE patients (3%) although lupus was one of the most frequent diagnosis associated with ANoA.

Staining patterns

Morphologically, there were three distinct nucleolar immunofluorescent patterns ie, speckled, homogeneous, and clumpy, as previously described by Bernstein *et al*[6] (Fig. 1). Homogeneous staining was the most commonly found nucleolar immunofluorescence (48/81) and was associated with SLE (24/48). Other diseases associated with this pattern included PSS (7), rheumatoid arthritis (4), Raynaud's disease (3), polydermatomyositis (3), discoid lupus (2), drug induced antinuclear antibodies (1), juvenile arthritis (1), primary Sjogren's (1), Reiter's syndrome (1) and polymyalgia rheumatica (1). Clumpy nucleolar staining was found in 18 patients. Eight of them had PSS. Three patients had lupus. Two patients had rheumatoid arthritis. One patient each had Raynaud's disease, drug induced antinuclear antibodies, juvenile arthritis, interstitial pulmonary fibrosis and Hashimoto's thyroiditis. It was interesting to note that almost all patients with speckle nucleolar staining (14/15) had PSS as their underlying disease. The only one lupus patient who had a speckle nucleolar pattern also had some features of scleroderma, ie Raynaud's phenomenon and sclerodactyly.

The organ manifestations in PSS patients with different nucleolar staining patterns were summarized in Table 3. Patients with clumpy nucleolar pattern showed significantly less pronounced female preponderance ($p=0.05$) and joint involvement ($p=0.00002$) than the ANoA-negative patients, but tended to have more common pulmonary manifestations ($p=0.055$). There was a trend towards more muscle involvement in patients with homogeneous patterns than those with no ANoA but with no statistical significant difference ($p=0.085$). We

Table 3. Comparison of scleroderma patients with different nucleolar staining patterns and those without nucleolar antibodies

Manifestations	With ANoA, n=29			Without ANoA, n=24
	Homo, n=7 (%)	Clumpy, n=18 (%)	Sp, n=15 (%)	(%)
Females	6 (85.7)	*12 (66.7)	15 (100)	22 (91.7)
Raynaud's phenomenon	6 (85.7)	17 (94.4)	13 (86.7)	22 (91.7)
Digital pitting scar	3 (42.9)	11 (61.1)	8 (53.3)	12 (50.0)
Telangiectasia	1 (14.3)	5 (27.8)	3 (20.0)	7 (29.2)
Kidney involvement	1 (14.3)	1 (5.6)	2 (13.3)	0
Lung involvement	5 (71.4)	+16 (88.9)	6 (40.0)	15 (62.5)
GI involvement	3 (42.9)	15 (83.3)	11 (73.3)	15 (62.5)
Muscle involvement	#4 (57.1)	5 (27.8)	1 (6.7)	5 (20.8)
Joint involvement	5 (71.4)	*3 (16.7)	11 (73.3)	20 (83.3)
Cardiac involvement	0	4 (22.2)	4 (26.7)	2 (8.3)

Homo = homogeneous nucleolar patterns,
Sp = speckle patterns,
* $p < 0.05$
+ $p = 0.055$
# $p = 0.085$
} versus patients without ANoA

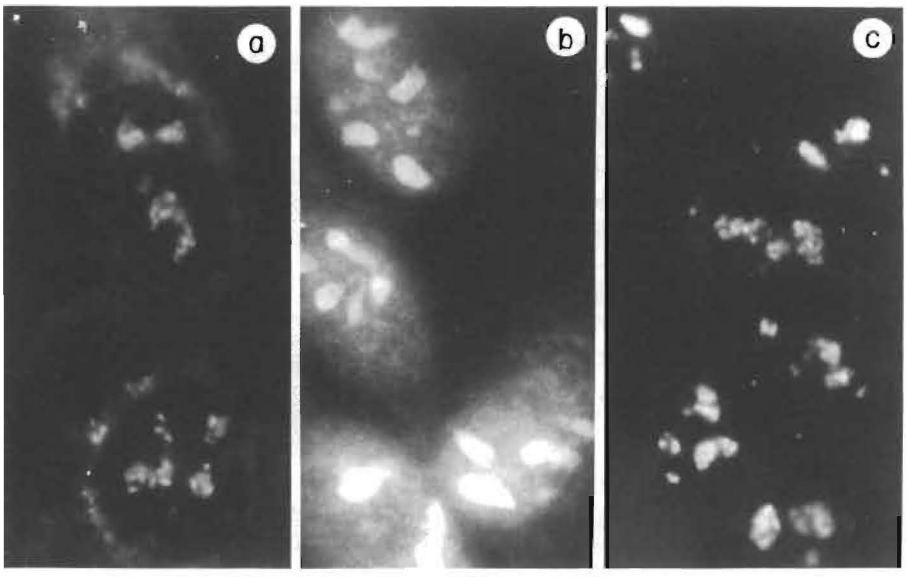

Fig. 1 Nucleolar immunofluorescent patterns using HEp-2 cells as substrate.
a) shows speckle nucleolar staining,
b) shows homogeneous or diffuse fluorescence of nucleoplasm and
c) shows clumpy nucleolar with condensed granules (× 100).

Table 4. Comparison of lupus patients with homogeneous nucleolar staining and those without antinucleolar antibodies*

Manifestations	with ANoA (%) n=24	without ANoA (%) n=119	P value
Female	17 (70.8)	113 (95)	0.0007
Mean age ± S.D.	32.5 ±15.2	27.4 ± 10.3	0.04
Skin involvement	24 (100)	107 (89.9)	0.09
Vasculitis	13 (54.2)	34 (28.6)	N.S.
Cardiopulmonary involvement	3 (12.5)	16 (13.4)	N.S.
Neuropsychiatric involvement	5 (20.8)	19 (16.0)	N.S.
Hematologic involvement	5 (20.8)	50 (42.0)	0.08
Renal involvement	19 (79.2)	87 (73.1)	N.S.
Arthritis/Arthalgia	11 (45.8)	67 (56.3)	N.S.
Myositis/Myalgia	9 (37.5)	22 (18.5)	0.07
Gastrointestinal involvement	2 (8.3)	8 (6.7)	N.S.

*AS3 and 1 lupus patients had clumpy and speckle nucleolar stainings, respectively. We choose to compare only patients with homogeneous staining and those without ANoA.

Table 5. ANoA positivity on different substrates

Substrate	Source	No. positive	% (compare to AI)
Tissue section	Mouse kidney	32	39.5
	Mouse liver	33	40.7
	Rat kidney	31	38.3
	Rat liver	32	39.5
Tissue culture	KB cells	75	95.6
	Kallestad HEp-2	79	97.5
	Bion HEp-2	77	95.1
	Immunoconcepts HEp-2	73	90.1
	Meloy HEp-2	35	43.2
	Antibodies Inc. HEp-2 (AI)	81	100

could not identify any particular clinical manifestation associated with the speckle staining in the scleroderma patients.

Almost all ANoA-positive lupus patients showed homogeneous nucleolar staining (24/28). They had significantly higher male sex ratio and age at onset than the ANoA-negative SLE patients ($p < 0.05$), and had less hematologic ($p = 0.08$) but more muscle ($p = 0.07$) and skin involvements ($p = 0.01$) than the comparison group.

Substrate sensitivity

Table 5 demonstrated ANoA positivity in 81 sera on different substrates. Compared to our best substrate (Antibodies, Inc.), ANoA positivity varied from 38% to 98% on 9 other substrates. All tissue sections were comparable in sensitivity for ANoA detection. Use of a cell culture substrate, either HEp-2 or KB cell, doubles the rate of positivity. However, this depends on appropriate selections of a commercial HEp-2 slides. While Kallestad, Bion and Immunoconcepts were comparable to Antibodies Inc, the Meloy slides showed poorest immunofluorescent staining and had not been better than the organ sections in detecting ANoA (Fig. 2).

DISCUSSION

Antinuclear antibody profiles have become diagnostically worthwhile. The patterns of nuclear immunofluorescence are not usually diagnostic but they serve as useful clues to the identity of the specific autoantibodies responsible for the nuclear immunofluorescence demonstrated by a particular serum. Nucleolar immunofluorescent patterns identify another class of autoanti-

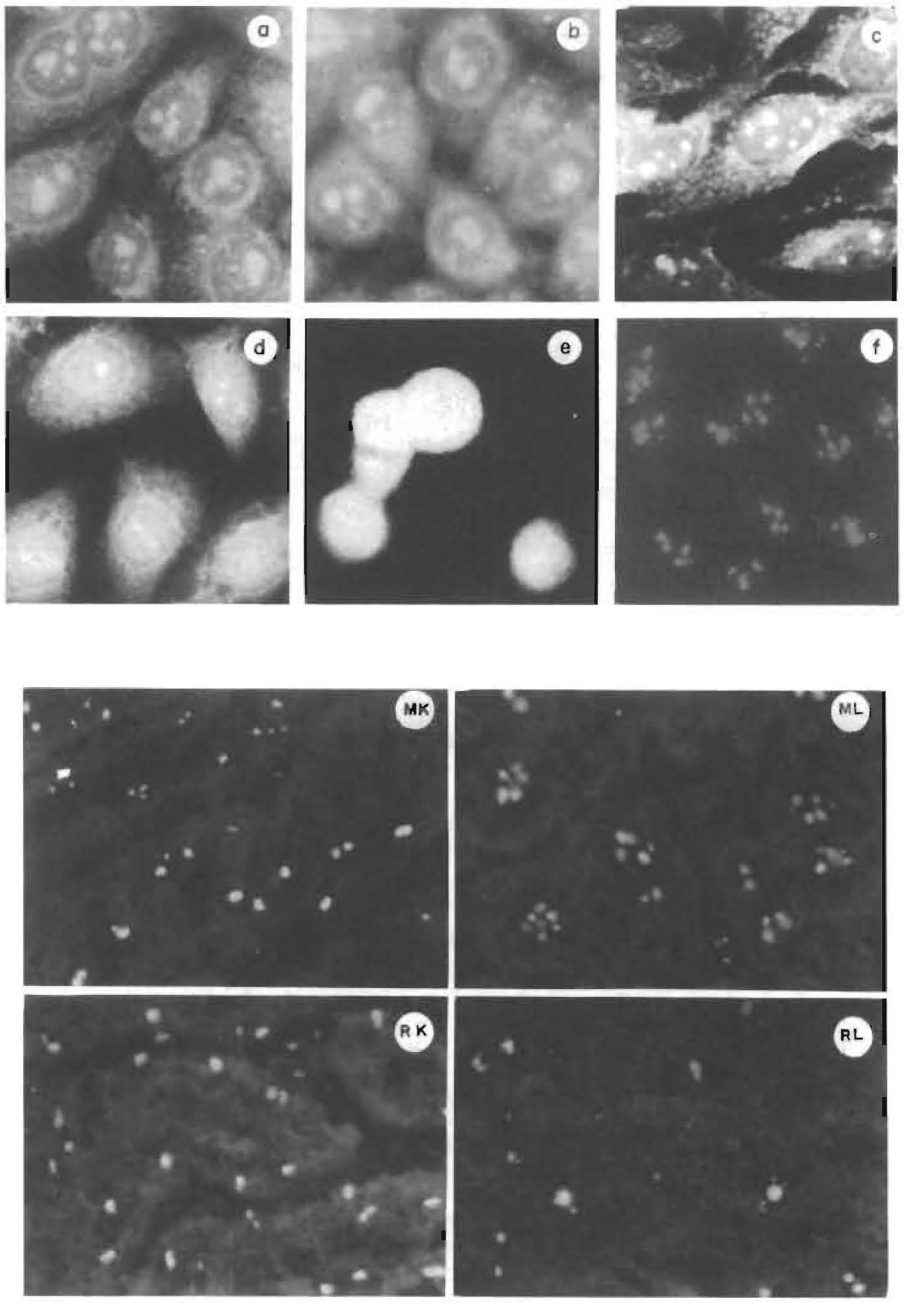

Fig. 2 Nucleolar staining on different substrates.
MK = mouse kidney, ML = mouse liver,
RK = rat kidney, RL = rat liver, a = Antibodies Inc. HEP-2,
b = Bion HEp-2, c = Kallestad HEp-2,
d = Immunoconcepts HEp-2, e = Meloy HEP-2,
f = KB cells (× 40).

bodies widely known to be associated with systemic sclerosis.[2-6] Our study demonstrated that ANoA are uncommon in unselected sera, but the use of a cell line substrate increases considerably the rate of positivity. Although the large size of nucleoli in the cell lines facilitates the detection of these antibodies, commercial tissue culture substrates are more sensitive than organ section substrates only when certain commercially available slides are selected. This was shown in our study when various commercially available HEp-2 substrates were compared.

We have also examined the diagnostic specificity of these antibodies. Although the majority of our patients who had these antibodies had either PSS or SLE as their underlying diseases, other non-rheumatic disorders such as Hashimoto's thyroiditis and interstitial pulmonary fibrosis were also associated with these antibodies. Unlike other nuclear immunofluorescent patterns,[9] nucleolar staining could not be demonstrated in normal human sera.

It was noted that speckle nucleolar staining with the exception of one serum appeared to occur exclusively in sera of patients with PSS. The single exception was in a patient who had a diagnosis of SLE. This patient had oral ulcers, arthritis, photosensitivity, and pericarditis together with some features of scleroderma, ie Raynaud's phenomenon and sclerodactyly. Reimer *et al*[10] showed that anti-RNA pol I antibodies in scleroderma sera produced speckle nucleolar staining. Thus, we conclude that these antibodies which are represented by speckle nucleolar immunofluorescence may be marker antibodies for the scleroderma-liked features.

Patients with clinically pure polymyositis have. been shown to have anti-PM-Scl antibodies.[11] These antibodies produce homogeneous nucleolar staining.[12] Although all of our polymyositis patients had homogeneous nucleolar patterns, most of the patients with this immunofluorescent staining had SLE as their underlying disease. Interestingly, both lupus and scleroderma patients who showed this nucleolar pattern had more muscle involvement than the comparison group. However, statistical analysis demonstrated only a trend and not a definite correlation between this nucleolar staining and the muscle pathology.

Clumpy nucleolar immunofluorescence was found in patients with non-rheumatic diseases such as Hashimoto's thyroiditis and interstitial pulmonary fibrosis. As these organs are commonly involved in PSS,[13,14] it may be possible that the autoantibodies producing this staining morphology are linked to the immunopathogenesis of thyroiditis and pulmonary fibrosis. Our study also demonstrated a trend towards more pulmonary involvement in scleroderma patients with this staining pattern.

In conclusion, identification of the nucleolar staining morphologies is helpful in confirming specific autoimmune diseases and predicting the clinical outcome. Unlike Western blotting and immunoprecipitation which can only be performed in research institutes, characterizations of the nucleolar staining patterns can be easily done at most general hospitals by an indirect immunofluorescence technique using appropriate tissue cultures as substrate.

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Preliminary Notice

Combined Inaugural Meeting of the Federation of Immunological Societies of Asia/Oceania (FIMSA)

and the

14th. Annual Scientific Meeting of the Australasian Society for Immunology

To be held in the Adelaide Convention Centre, Adelaide, South Australia, Australia.

From the 1st. to 6th. of December, 1996.

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This meeting will feature an array of keynote addresses presented by leading international scientists.

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