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Anti-fibrillarin autoantibodies in mercury-treated mice

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SUMMARY

Using indirect immunofluorescence (IF) with HEp-2 cells as a substrate serially bled SJL mice were found to gradually develop a high titre of anti-nucleolar antibodies (ANuA) after 3–5 weeks of s.c. injections of 1·6 mg $HgCl_2$/kg body weight every third day. The ANuA showed a clumpy nucleolar pattern of localization and were composed of all IgG subclasses, but contained, in comparison with the antinuclear antibodies (ANA) in MRL-1pr/1pr mice, significantly lower titres of IgG2a and only traces of IgG3. Immunoblotting analysis using purified mouse liver nucleoli revealed that the sera with ANuA identified the same 34-kD nucleolar protein which was targeted by a human scleroderma serum containing autoantibodies monospecific for fibrillarin. In addition, a fraction of the mercury-treated SJL mice developed serum antibodies reacting with 10–15 and 60–70 kD nucleolar proteins in immunoblotting. The presence of serum autoantibodies reacting with the 10–15 kD proteins correlated with significantly increased titres of anti-histone antibodies of the IgG class in ELISA. Some mercury-treated SJL mice also developed a significantly increased titre of anti-histone antibodies of the IgM class. B10.S mice treated with mercuric chloride consistently developed ANuA, which also targeted a 34-kD nucleolar protein. Since anti-fibrillarin antibodies are specific markers of scleroderma, the present animal model may be valuable for studies of the immunological aberrations which are likely to induce this autoimmune response.

Keywords mercury mice anti-nuclear antibodies immunoglobulin isotypes scleroderma

INTRODUCTION

Serum anti-nuclear antibodies (ANA) are a hallmark of systemic autoimmune diseases, and the development of more sensitive methods for their detection has led to an increased awareness of the autoimmune component in certain diseases (Tan *et al.*, 1988). This is well exemplified by progressive systemic sclerosis (scleroderma), which is a generalized disorder of the connective tissue characterized by a fibrotic thickening of the skin and synovia, intimal thickening of the arteries, and a variable involvement of the internal organs. A generalized form with diffuse cutaneous thickening, widespread visceral involvement, and usually a rapidly fatal course has been termed diffuse scleroderma, whereas the CREST syndrome (Calcinosis, Raynaud's phenomenon, Esophage dysfunction, Sclerodactyly and Telangiectasia) denotes a variant with primary involvement of the skin in the face and the fingers and of the oesophagus but with extension to other internal organs only after a protracted course (Alarcon-Segovia, 1985).

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Although ANA were reported already in the 1960s to occur in many of the scleroderma patients (Swanson-Beck *et al.*, 1963; Rothfield & Rodnan, 1968), the recent use of more sensitive methods, especially tissue culture cells as substrate in indirect immunofluorescence (IF), has revealed that ANA occur in virtually all patients with scleroderma (Tan *et al.*, 1980). The specificity of these antibodies includes the antibody to the Scl-70 antigen, a 70-kD degradation product of DNA topoisomerase I (Guldner *et al.*, 1984) occurring in most of the patients with diffuse scleroderma (Jarzabek-Chorzelska *et al.*, 1986), and antibodies to the centromere/kinetochore antigens which are highly selective for the CREST syndrome (Bernstein, Steigerwald & Tan, 1982; Earnshaw & Rothfield, 1985). Another group of autoantibodies is the anti-nucleolar antibodies (ANuA), which occur in a low frequency in most connective tissue syndromes (Maini, Charles & Venables, 1985), but have been found in 8–47% of sera from patients with scleroderma (Tan *et al.*, 1980; Bernstein *et al.*, 1982; Riboldi *et al.*, 1985; Reimer *et al.*, 1987a; 1988). Several antigens have been identified which might relate to a nucleolar staining in indirect IF including a 4–6 S RNA (Pinnas, Northway & Tan, 1973); a 7-2 RNA complexed with proteins (To-antigen) (Reddy *et al.*, 1983);

RNA polymerase I (Reimer *et al.*, 1987a); the PM/Scl antigen (Reimer *et al.*, 1986); and a U3-ribonucleoprotein (RNP) particle (Reddy *et al.*, 1983). The specific target for the ANuA directed against the U3-RNP is a 34-kD, basic (pI 8·5) nucleolar protein named fibrillarin due to its localization in the fibrillar region of the nucleolus (Lischwe *et al.*, 1985; Ochs *et al.*, 1985). In a recent study, fibrillarin was found to be the antigen for approximately half of the high-titre IgG class ANuA in scleroderma patients (Reimer *et al.*, 1988).

Although scleroderma-like syndromes develop spontaneously in several disease conditions (Alarcon-Segovia, 1985) and after exposure to chemicals such as vinyl chloride (Lilis *et al.*, 1975), the only model with a defined immunological basis is the scleroderma-like syndrome seen in experimental chronic graft-*versus*-host disease (GVHD) (Jaffe & Claman, 1983; Gelpi *et al.*, 1988). The occurrence of ANuA seems to be rare in murine models of lupus-like disease (Theofilopoulos & Dixon, 1985), and the lack of a suitable experimental model is likely to have hampered the further understanding of ANuA pathogenesis and, therefore, a closer understanding of the immunological aspects of scleroderma. In the present study two strains of mice have been shown to consistently develop IgG anti-fibrillarin antibodies after treatment with the simple chemical mercuric chloride, and this model should facilitate further studies on the mechanisms underlying development of ANuA.

MATERIALS AND METHODS

Experimental procedure

Female SJL/N mice were obtained from G1 Bomholtgaard (Ry, Denmark) and female B10.S and MRL-1pr/1pr (MRL) mice from Harlan Olac (Bicester, UK). All mice were 8–10 weeks old at the beginning of the experiments, except for the MRL mice which were 5–6 months old when killed. SJL and B10.S mice were treated with a s.c. injection of either 1·6 mg $HgCl_2$/kg body weight or 0·1 ml of a sterile saline solution every third day for up to 10 weeks. The MRL mice were not treated.

Serum ANA test

Indirect IF microscopy was employed using HEp-2 cells (Kallestad Lab., Austin, TX) as a substrate, serum diluted 1:20 with phosphate-buffered saline (PBS) (pH 7·4) as the first-step reagent, and FITC-conjugated goat anti-mouse IgG and IgM antibodies (Jackson Immunoresearch Lab., West Grove, PA) diluted 1:20 with PBS as the second-step reagent (Hultman & Eneström, 1987). The fluorescence intensity of ANA was assessed without knowledge of strain or treatment given and graded as negative (0); weak (+1); moderate (+2); strong (+3); and very strong (+4).

Serum ANA isotype profile

Serum pooled from nine SJL mice treated with $HgCl_2$ for 4–5 weeks, and serum pooled from five MRL mice, 5–6 months old, were analysed by chess-board titration using serial dilutions of serum as the first step reagent, HEp-2 cells as a substrate, and FITC-conjugated goat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 (Southern Biotechnology, Birmingham, AL) antibodies with a fluorescein/protein ratio of 5·3, 4·3, 4·5, and 4·8, respectively, as the second-step reagent.

Anti-histone antibody ELISA

IgG and IgM anti-histone antibodies were detected as described by Rubin (1986). Mouse sera, diluted 200-fold, were added to ELISA plates coated with total calf thymus histone. Following a 2-h incubation plates were washed with PBS–Tween and anti-mouse immunoglobulin detecting reagents were added for a 2-h incubation. Plates were washed before substrate addition and OD_{405} was measured after 1 h. Background values, obtained from antigen-coated wells in the absence of serum, were subtracted from test values. The specificity of the immunoglobulin class-specific detecting reagents (Caltag Laboratories, San Francisco, CA) was confirmed with IgM and IgG monoclonal anti-histone antibodies.

Immunoblotting

Nucleoli were isolated from fresh mouse liver as previously described (Reimer *et al.*, 1987b). Electrophoretic transfer of SDS–PAGE fractionated nucleolar proteins to nitrocellulose was performed as described by Towbin, Staehelin & Gordon (1979) with minor modifications. Following SDS–PAGE, electrotransfer to nitrocellulose was performed at 4°C for 3 h at 60 V. Nitrocellulose strips were blocked in a solution of 3% non-fat powdered milk in PBS–0·05% Tween 20 for 60 min before being overlayed with serum diluted 100-fold in PBS–Tween–non-fat milk. Bound antibody was detected with ^{125}I-labelled protein A (New England Nuclear, Boston, MA), followed by autoradiography at −70°C.

RESULTS

Serum ANA in SJL mice

ANuA of the IgM or the IgG class were not seen in SJL mice before treatment with mercuric chloride began, and did not develop in the saline-treated controls (Fig. 1a, Table 1). In contrast, serum ANuA of the IgG class developed in all 11 mercuric chloride-treated mice (Fig. 1a), and three mice also showed ANuA of the IgM class but with a low fluorescence intensity (Table 1). The nucleolar staining pattern of both the IgM and the IgG antibodies was clumpy according to the terminology used by Bernstein *et al.* (1982). Five of the mercury-treated SJL mice developed, in addition, a relatively weak homogeneous ANA pattern which was exclusively of the IgG class (Fig. 1b). Two other SJL mice in the mercury-treatment group showed ANA of the IgG class with a homogeneous pattern before treatment with mercuric chloride began (Fig. 1b). All mercury-treated SJL mice had serum ANA of the IgG class giving a dot-like staining pattern in the nucleoplasm (Fig. 2). Serum from one of the five SJL control mice contained IgG ANA with a homogenous pattern before treatment with saline began, whereas the other controls showed no ANA neither before nor after saline treatment (Table 1).

Serum ANA isotype profile

ANuA of all IgG subclasses were found in the pooled serum from SJL mice treated with mercuric chloride for 4–5 weeks (Figs 2a–c; 3). The pooled serum from aged MRL mice showed a speckled ANA pattern (Fig. 2d–f) which consisted of all IgG subclasses, but with a predominance of IgG2a (Fig. 3). The SJL serum contained, in comparison with the speckled ANA in the serum from aged MRL mice, ANuA with five steps lower titre of

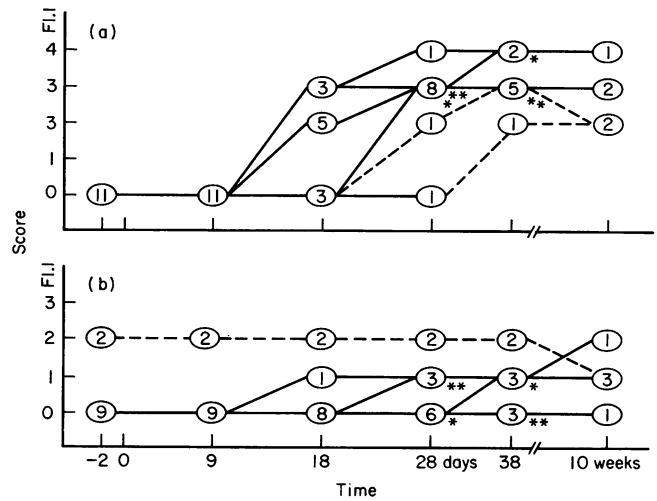

Fig. 1. Kinetics of serum antinuclear antibodies in SJL mice given s.c. injections of mercuric chloride beginning at t = 0. Indirect immunofluorescence using HEp-2 cells as a substrate, serum diluted 1:20 as the first-step reagent, and FITC-conjugated goat anti-mouse IgG diluted 1:20 as the second-step reagent. Grading of fluorescence intensity: 0 = negative; +1 = weak; +2 = moderate; +3 = strong; and +4 = very strong. (a) Clumpy nucleolar pattern; (b) Homogeneous nuclear pattern. Figures within circles denote the number of animals with the specified fluorescence at the specified point of time. Stars represent the number of killed animals. (- - -), Fluorescence intensity of ANuA (a) and homogeneous ANA (b) in sera from the two mice (F3, F6) which had a homogeneous ANA pattern before mercury treatment.

IgG2a and only traces of IgG3 (Fig. 3). Traces of ANuA of the IgM class were also seen in the pooled serum from mercuric chloride-treated SJL mice (data not shown).

Serum ANA in B10.S mice

All eight mercuric chloride-treated B10.S mice showed serum IgG autoantibodies with a clumpy nucleolar pattern and a dot-like staining in the nucleoplasm. The B10.S controls showed no ANuA. Five of the mercuric chloride-treated B10.S mice also developed ANA of the IgG class with a homogenous pattern and of low intensity. However, a similar ANA pattern was also seen in sera from three of the seven B10.S controls (data not shown).

Anti-histone antibody ELISA

Three of the seven mercuric chloride-treated SJL mice examined had significantly increased serum titres of IgG antihistone antibodies, and these sera came from mice which developed a homogenous ANA pattern of the IgG class during mercury treatment (Table 1). In contrast, the two SJL mice which had a homogeneous IgG ANA before treatment with $HgCl_2$ began (F3 and F6) did not show increased serum anti-histone antibody titres neither before nor after mercury treatment. Three mercury-treated mice had modestly increased titres of serum IgM anti-histone antibodies. None of the four SJL controls examined had antihistone antibodies. Sera from both a mercuric chloride-treated and a control B10.S mouse contained low but significantly increased titres of IgM anti-histone antibodies, whereas only serum from the mercuric chloride-treated B10.S mouse had significantly increased IgG anti-histone antibody titre (Table 1). When sera were assayed without coating the wells with histone, detection using anti-IgM and anti-IgG antibodies gave no background values.

Immunoblotting

Six of the seven sera from mercury-treated SJL mice contained antibodies which reacted with a 34-kD nucleolar protein (Fig. 4; Table 1). This protein was also targeted by a scleroderma reference serum (S4) (Fig. 4) earlier shown to contain antibodies monospecific for fibrillarin (Reimer *et al.*, 1987b). The single serum (F6) which did not react with the 34-kD protein came from a mouse which had a homogeneous serum ANA pattern before treatment with mercury began; showed a delayed development of ANuA during mercury treatment; and attained the lowest serum ANuA titre during mercury treatment (Fig. 1, Table 1). Four of the sera from mercury-treated SJL mice reacted with low mol. wt protein(s) of 10–15 kD also (Fig. 4), and sera from three of these mice showed high titres of IgG anti-histone antibodies (Table 1). Sera from four mercuric chloride-treated SJL mice reacted with one or more nucleolar protein(s) with a molecular weight of 60–70 kD (Fig. 4; Table 1). The serum from a mercury-treated B10.S mouse reacted only with the 34-kD protein, whereas the serum from a control B10.S mouse showed no reactivity to nucleolar proteins in immunoblotting (Fig. 4).

DISCUSSION

While this work was in progress, Reuter *et al.* (1989) reported the finding of autoantibodies to fibrillarin in B10.S mice treated with mercuric chloride. They were not able to detect autoantibodies of other specificities although they did not exclude the possibility of their existence. In this report, SJL mice of a similar H-2 haplotype ($H\text{-}2^s$) are clearly shown to develop autoantibodies of several specificities one of which is fibrillarin with a second group directed against histones. Yet a third group of autoantibodies was detected, which reacted with antigens in the 60–70 kD mol. wt range, which have not been characterized. Thus, this experimental model of autoimmunity should not be regarded as a restricted autoimmune response to the nucleolar

Table 1. Serological findings in $HgCl_2$- and NaCl-treated mice

Mice		FANA test*				Titre of antihistone antibodies†		
		IgG		IgM				
Treatment‡	N:o	Hom.	Nuo.	Hom.	Nuo.	IgG	IgM	Immunoblotting§
SJL								
NaCl 5w	E1	—	—	—	—	0·007	0	—
	E2	—	—	—	—	0	0	—
	E3	1	—	—	—	0·004	0	—
	E4	—	—	—	—	ND	ND	ND
	E5	—	—	—	—	0·014	0·005	—
Pretreatment	F1	—	—	—	—	0·047	0·080	—
$HgCl_2$ 5 w	F1	—	3	—	—	0·056	*0·194*	34
$HgCl_2$ 5 w	F2	2	4	—	1	*0·143*	*0·514*	L, 34, H
Pretreatment	F3	2	—	—	—	0·082	0·108	—
$HgCl_2$ 5 w	F3	2	3	—	—	0·062	0	34, H
Pretreatment	F4	—	—	—	—	0	0	—
$HgCl_2$ 5 w	F4	1	3	—	2	*1·589*	*0·199*	L, 34, H
Pretreatment	F6	2	—	—	—	0	0	—
$HgCl_2$ 5 w	F6	2	2	—	—	0·016	0	L
Pretreatment	G1	—	—	—	—	0	0	—
$HgCl_2$ 4 w	G1	—	3	—	—	0·050	0	34
$HgCl_2$ 5 w	G5	1	4	—	2	*1·605*	0·004	L, 34, H
B10.S								
NaCl 5 w	A	—	—	—	—	0·012	*0·191*	—
$HgCl_2$ 5 w	B	—	3	—	—	*0·178*	*0·199*	34

* FANA, fluorescence anti-nuclear antibody test, using serum diluted 1:20, HEp-2 cells, and FITC-conjugated goat anti-mouse IgM and IgG. —, negative; 1, weak intensity; 2, moderate intensity; 3, strong intensity; 4, very strong intensity. Hom., homogeneous staining pattern; Nuo., nucleolar staining pattern.

† ELISA test using total calf thymus histones, peroxidase-conjugated GAM IgM and IgG. Titres are OD_{405} after subtraction of background values. Significantly increased titres are italicized. ND, not determined.

‡ NaCl, s.c. injection of 0·1 ml 0·9% NaCl every third day; $HgCl_2$, s.c. injection of 1·6 mg $HgCl_2$/kg every third day. w, weeks.

§ Western blotting using mouse liver nucleolar proteins followed by incubation with mouse serum and detection of bound antibody with ^{125}I-protein A followed by autoradiography. The molecular weights of the detected bands are indicated. L, 10–15 kD; 34, 34 kD; H, 60–70 kD.

and U3-RNP-associated protein fibrillarin alone. Nevertheless, considering the kinetics of the immune response as displayed in Fig. 1, induction of autoantibodies to fibrillarin appears to be more vigorous and consistent than to other antigens. It is possible that other genetic factors besides the H-2 haplotype may determine the total nature of the immune response. Another feature of the SJL immune response is the relatively low titre of autoantibodies of IgG3 and IgG2a subclasses, in contrast to the autoimmune response in MRL-lpr/lpr mice which involves all IgG subclasses with a predominance of IgG2a (Eisenberg, Craven & Cohen, 1987; Fisher, Eisenberg & Cohen, 1988; and the present report).

ANuA occur in a subset of patients with scleroderma, and the nucleolar antigens which have been identified include fibrillarin, RNA polymerase I, a protein complex of many subunits called PM-Scl, and a nucleolar RNP particle containing 7-2 RNA (Tan *et al.*, 1988). Approximately one-half of the group of patients with ANuA has anti-fibrillarin antibodies (Reimer *et al.*, 1988), and autoantibody of this specificity appears to be scleroderma-specific. Hence, the mercuric chloride animal model may be of special interest in view of the induction of anti-fibrillarin autoantibodies and the possibility that the model might provide insights into disease mechanisms in scleroderma. However, the mercuric chloride model also results in the induction of autoantibodies to histones, a phenomenon which has been reported only rarely in scleroderma (Gioud, Kaci & Monier, 1985). In this respect, the mercuric chloride induced autoimmune response resembles the lupus-like syndrome induced by chemicals (drugs), such as procainamide and hydralazine, in which antihistone antibodies are a special characteristic (Portanova *et al.*, 1982; Totoritis *et al.*, 1988). The mechanism of drug-induced lupus is unclear, although it has been attributed to an effect of drug metabolites causing cell death with the release of histones providing a potential for an antigen-driven development of autoantibodies (Rubin, Uetrecht & Jones, 1987). At least in the SJL mouse strain there

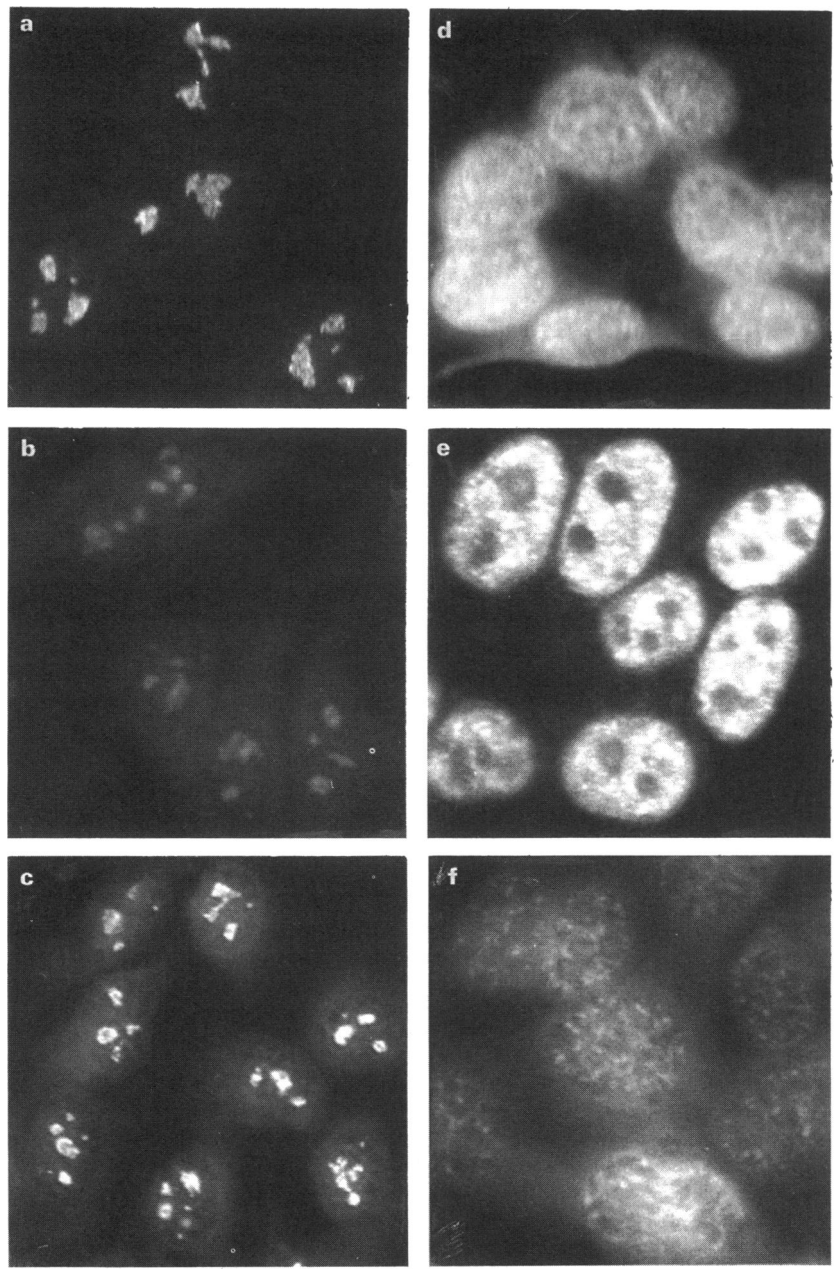

Fig. 2. Anti-nuclear antibody (ANA) pattern in mercury-treated SJL mice and aged MRL mice. Serum ANA using HEp-2 cells as a substrate, serum diluted 1:20 as the first-step reagent, and FITC-conjugated goat anti-mouse immunoglobulin antibodies diluted 1:20 as the second-step reagent. a–c, Pooled serum from nine SJL mice after 4–5 weeks mercury treatment showing a clumpy nucleolar staining and a nuclear dot-like staining. (a) IgG1, × 540; (b) IgG2a, × 880; and c) IgG2b, × 750. d–f, Pooled serum from five MRL mice aged 5–6 months; (d) IgG1, finely speckled staining, × 750; (e) IgG2a, coarsely speckled staining, × 950; and (f) IgG2b, finely speckled staining, × 880.

appears to be a coupling of the specificities in the mercury-induced immune response involving fibrillarin, histones and certain high mol. wt proteins. SJL mice develop initially during mercuric chloride-treatment a polyclonal B cell activation, which is suppressed after 4 weeks despite ongoing mercury treatment (Hultman & Enestrom, 1989). Although the multiple immune response specificities observed in this study may reflect this initial, broad B cell activation, the question is still how these antigens are related to each other since mercuric chloride for yet unknown reasons elicits a restricted autoimmune response.

Besides the T cell dependency of protein antigens in general (Jones, 1987), the dominance of the IgG class in the serum ANuA (present report) and the linkage of the development of ANuA to the H-2 complex (Robinson, Balazs & Egorov, 1986; Gleichmann *et al.*, 1988; Hultman and Enestrom, unpublished observations) strongly suggest that the immune response to fibrillarin in mercury-treated mice is dependent on T cells. Such a T cell dependent immune response might theoretically involve T helper cells specific or unspecific for fibrillarin. There is no experimental support for the existence of T helper cells specific

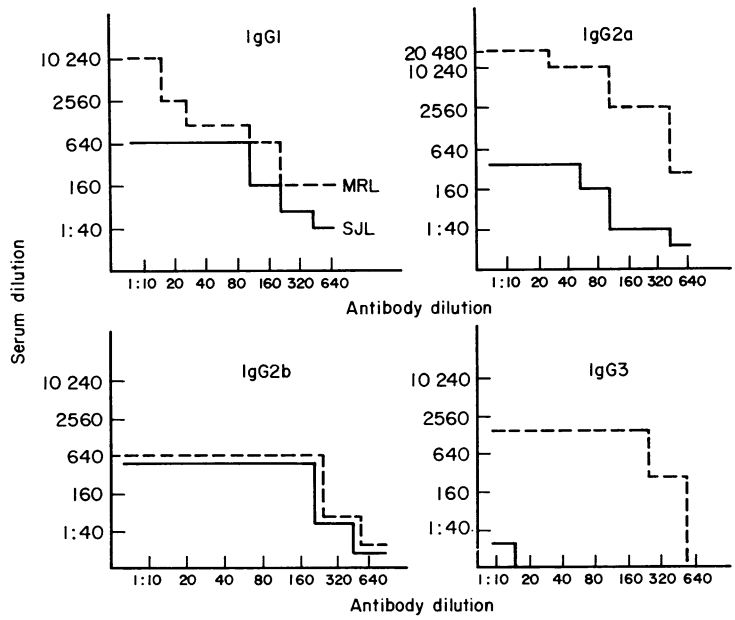

Fig. 3. IgG subclass profile of serum antinuclear antibodies in mercury-treated SJL mice and aged MRL mice. Chess-board titration using HEp-2 cells as a substrate, serially diluted pooled serum from mercury-treated SJL mice ($n=9$) or aged MRL mice ($n=5$) as the first-step reagent, and serially diluted FITC-conjugated goat antibodies to the different mouse IgG subclasses as the second step reagent.

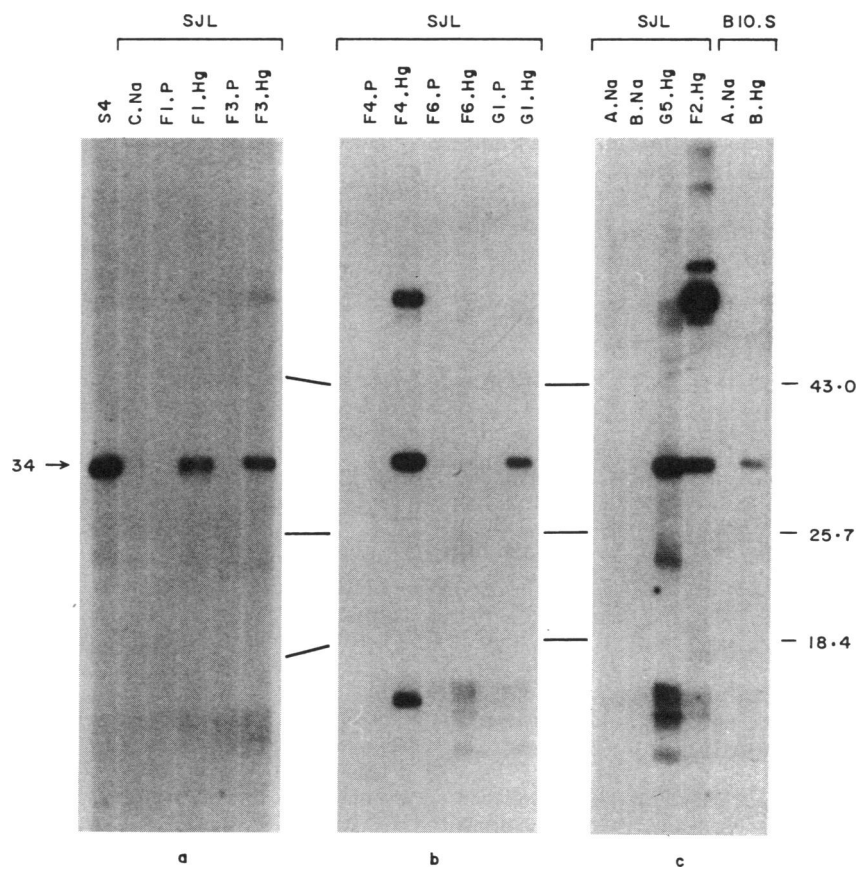

Fig. 4. Immunoblotting of SJL and B10.S mice serum on mouse liver nucleoli following SDS–PAGE. (a) Prototype human anti-fibrillarin serum (S4), serum from a NaCl-treated SJL mouse (C.Na), and sera from mice F1 and F3 pre-(P) and post-(Hg) mercuric chloride treatment; (b) immunoblotting using sera from three additional SJL mice pre- and post-mercuric chloride treatment; (c) the result of immunoblotting using sera from two SJL mice treated with NaCl (A.Na; B.Na); two SJL mice treated with mercuric chloride (G5.Hg;F2.Hg); and two B10.S mice, one treated with NaCl (A.Na) and one with mercuric chloride (B.Hg).

for fibrillarin. However, anti-fibrillarin antibodies have recently been demonstrated in a fraction of mice with SLE-like chronic GVHD (Gelpi *et al.*, 1988), a condition in which alloreactive T helper cells without antigen-specificity are believed to be involved (Rolink & Gleichmann, 1983). Mercuric chloride-induced autoimmunity and autoimmunity due to SLE-like chronic GVHD have features in common besides the T cell dependency. Both share similar autoantibody specificities (i.e. anti-fibrillarin and antihistone) although in the case of the chronic GVHD model it is the antihistone autoantibodies that predominate (Portanova, Claman & Kotzin, 1985; Pollard *et al.*, 1987). However, the high titre of ANuA persisting for at least 12 weeks in mercury-treated SJL mice (Hultman & Enestrom, 1988), reflects a continuous clonal expansion of B cells with anti-fibrillarin specificity in this animal model. In chronic GVHD it is thought that the availability of antigen (i.e. DNA and histones) in the correct form is the stimulus for the restricted autoantibody response (Gleichmann *et al.*, 1984; Pollard *et al.*, 1987). In the case of mercuric chloride-induced autoimmunity the predominance of the anti-fibrillarin response could arise from mercury-induced cell damage which would favour the presentation of nucleolar material to B cells. Proliferation of such B cells could be brought about by lymphokines released from T cells under the stimulatory effects of mercuric chloride (Reardon & Lucas, 1987).

A detailed analysis of the effects of mercuric chloride on cellular structure and function, focusing in particular on the nucleolus, may be rewarding if we are to understand the specific autoimmune response elicted by this toxin. This opinion is influenced in part by the exclusive nucleolar localization of fibrillarin and the known presence of histones in the nucleolus. Additional sera from B10.S mice and other mouse strains which develop ANuA during mercury treatment will have to be analysed in order to see if genetic factors other than the H-2 complex determine the specificity of the mercury-induced ANuA.

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