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Immunology

Mercuric chloride induces autoantibodies against U3 small nuclear ribonucleoprotein in susceptible mice

(autoimmunity/scleroderma/nucleolar autoantibodies)

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ABSTRACT **Autoantibodies to nucleolar components are a common serological feature of patients suffering from scleroderma, a collagen vascular autoimmune disease. While animal models, which spontaneously develop abundant anti-nucleolar antibodies, have not yet been described, high titers of such antibodies may be induced by treating susceptible strains of mice with mercuric chloride. We have identified the nucleolar autoantigen against which the $HgCl_2$-induced IgG autoantibodies from mice of strain B10.S are directed. It is a protein with an apparent molecular mass of 36 kDa and a pI value of ≈8.6, which is associated with the nucleolar small nuclear RNA U3, and by these criteria must be identical with a polypeptide called fibrillarin. It is striking that scleroderma patients spontaneously produce autoantibodies against the same U3 ribonucleoprotein (RNP). The $HgCl_2$-induced murine and the scleroderma-specific human anti-U3 RNP autoantibodies were indistinguishable in their reactivities toward fibrillarin. They further resemble each other insofar as both recognize epitopes on the 36-kDa protein, which have been highly conserved throughout evolution. Our results provide a basis to investigate at the molecular level whether similar immunoregulatory dysfunctions may lead to the preferential anti-U3 RNP autoantibody production in the animal model and in scleroderma patients.**

Autoantibodies against the nucleolus are produced spontaneously by patients with collagen vascular diseases and, among such patients, are associated particularly with progressive systemic sclerosis (scleroderma) (1–3). Sera from such patients stain nucleoli in distinctive patterns, indicating the presence of autoantibodies against a variety of nucleolar components. During recent years the identity of several of these nucleolar autoantigens has been described in molecular terms; they include the PM/Scl antigen (4, 5), RNA polymerase I (6, 7), a 90-kDa protein of the nucleolus-organizing region (8), as well as small nucleolar ribonucleoproteins (RNPs), the 7-2 RNP or To RNP (9, 10), and the U3 small nuclear RNP (snRNP) (10). Autoantibodies against U3 snRNP were shown to react with a basic protein of pI 8.5 and with a molecular mass between 34 and 36 kDa (11, 12). This protein was originally termed fibrillarin, since it is located in the fibrillar compartment of the nucleolus (13).

Despite this progress in the characterization of the nucleolar autoantigens, little is known about the mechanisms that lead to formation of the anti-nucleolar autoantibodies. This might be due partly to the lack of appropriate animal models. While murine models, such as NZB/W, MRL/1, or BXSB mice, which spontaneously develop immunopathies resembling the human collagen vascular diseases, have contributed considerably to the understanding of various immunological abnormalities involved in the pathogenesis of these syndromes, production of anti-nucleolar autoantibodies is not a prominent feature of the spontaneous models.

In humans, some of the systemic autoimmune diseases can be induced by a number of drugs or chemicals. For instance, procainamide and hydralazine may cause a form of systemic lupus erythematosus (SLE) that resembles idiopathic SLE both clinically and serologically. Moreover, exposure to the occupational chemical vinyl chloride or the drug bleomycin can result in a disorder resembling idiopathic scleroderma. In both cases, a genetic predisposition appears to be necessary for induction of the pathological manifestations. For example, patients with vinyl chloride-induced scleroderma show an elevated frequency of HLA-DR5 and an increased linkage disequilibrium between HLA-B8 and HLA-DR3 similar to idiopathic scleroderma patients (14).

From the use of mercury as a constituent of certain drugs and cosmetics and from occupational exposure it is known that subtoxic amounts of mercurials can cause immune complex glomerulonephritis in humans (15, 16). In susceptible strains of rat (17–19), subtoxic doses of mercuric chloride ($HgCl_2$) cause immune complex glomerulopathies (17–19), often associated with autoantibodies to DNA and other anti-nuclear antigens (18, 20, 21). When $HgCl_2$ was administered to susceptible strains of mice, they developed high titers of autoantibodies against nucleolar antigens (22–24). In highly susceptible mouse strains, such as B10.S, this was accompanied by granular deposits of IgG in the glomeruli and vessel walls (24) similar to the picture seen in scleroderma. The response to treatment with $HgCl_2$ is determined by at least two different loci, one of which is located in *H-2*, the murine major histocompatibility complex: the haplotype $H\text{-}2^s$ encodes susceptibility, while $H\text{-}2^a$ and $H\text{-}2^d$ determine resistance (23, 24). Within the $H\text{-}2^s$ haplotype, are class II major histocompatibility complex loci—i.e., A_β and/or A_α—that determine the susceptibility to $HgCl_2$ (ref. 23; E.G., unpublished results).

We were interested in identifying the nucleolar antigens against which strain B10.S ($H\text{-}2^s$) produces autoantibodies upon treatment with $HgCl_2$. By immunoblot analysis, a single 36-kDa protein of pI 8.6 was shown to be antigenic for the Hg-induced sera. In two-dimensional electrophoresis, it was indistinguishable from fibrillarin, which is associated with U3 small nuclear RNA (snRNA) and against which scleroderma patients may develop autoantibodies. Furthermore, both scleroderma patient serum and Hg-induced murine sera

Abbreviations: RNP, ribonucleoprotein; snRNA, small nuclear RNA; snRNP, small nuclear RNP; m^3G, 2,2,7-trimethylguanosine.
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selectively only precipitated U3 RNP from a cellular extract. We therefore conclude that $HgCl_2$-treated B10.S mice preferentially develop autoantibodies against the 36-kDa protein fibrillarin, an autoantigen that is also recognized by sera from scleroderma patients.

MATERIALS AND METHODS

Mice. Female B10.S (*H-2*^{s}) mice, specific pathogen-free, were purchased from OLAC 1976 Ltd. (Bicester, Oxon, U.K.) and kept under specific pathogen-free conditions. Mice were 8–10 weeks old at the start of the experiment.

Treatment of Mice. A 0.1% stock solution of $HgCl_2$ (analytical grade, Merck) was prepared in sterile pyrogen-free water. Before use, the stock solution was diluted 1:10 in sterile pyrogen-free saline. $HgCl_2$ (0.01 mg per mouse) was injected s.c. thrice weekly until week 12. Control mice received injections of saline only. Blood was taken from the retroorbital plexus at the times indicated.

Indirect Immunofluorescence. Immunofluorescence studies with mouse sera were performed either on unfixed cryostat sections (4 μm thick) of liver tissue from a rabbit or on mouse 3T3 fibroblasts grown on coverslips and fixed by methanol and acetone as described (25).

Immunoblot. A nuclear extract of HeLa S3 or Ehrlich ascites tumor cells was prepared and used for immunoblotting studies essentially as described (26). Affinity purification of mouse antibodies on preparative immunoblots of total nuclear proteins from HeLa cells was carried out as described by Smith and Fisher (27).

Two-Dimensional Gel Electrophoresis and Double-Label Immunoblot. HeLa nuclei were isolated and digested by RNase A and DNase I (26). Proteins were precipitated with acetone and subjected to nonequilibrium pH gradient gel electrophoresis in the first dimension (28) and to electrophoresis in a 15% SDS/polyacrylamide gel in the second dimension. The proteins in the gel were transferred electrophoretically to nitrocellulose, on which they were visualized by Ponceau S (Serva) so that the position of the major heterogeneous nuclear RNP proteins A1, A2, B1a, B1b, and B2 could be identified. The antibody reaction was performed as described above for one-dimensionally separated proteins.

Immunoprecipitation, 3′-End Labeling, and Urea Gel Electrophoresis. A cell extract (low speed) was prepared from HeLa S3 cells essentially as described by Parker and Steitz (12) except that the cells were sonicated in NE-4 (10 mM Tris·HCl, pH 7.4/100 mM NaCl/1 mM $MgCl_2$/1 mM phenylmethylsulfonyl fluoride/1 mM dithioerythritol) at a density of 1×10^8 cells per ml. Alternatively, the HeLa cell extract (high speed) was freed from preribosomal particles by an additional centrifugation at 120,000 × *g* for 2 hr (10). The procedure of immunoprecipitation was based on that of Matter *et al.* (29). Protein A-Sepharose CL-4B (2 μg) (Pharmacia), preswollen in 500 μl of NET-2 (40 mM Tris·HCl, pH 7.5/150 mM NaCl/0.05% Nonidet P-40), was incubated with 10 μl of ammonium sulfate-fractionated serum overnight at 4°C and then washed three times with NET-2. The antibody-bound beads were then incubated for 2 hr at 4°C with 100 μl of the cell extract and 400 μl of NET-2. After six washes with NET-2, the beads were extracted with phenol and the nucleic acids were precipitated by ethanol and either directly 3′-end-labeled with [^{32}P]pCp and RNA ligase (30) (Biolabs, North Brook, IL) or immunoprecipitated with 50 μg of rabbit anti-2,2,7-trimethylguanosine (m^3G) cap IgG essentially as described above, and then 3′-end-labeled. All RNA samples were separated by electrophoresis on 7 M urea/10% polyacrylamide (29:1, acrylamide/bisacrylamide) gels with 0.5× TBE (45 mM Tris base/45 mM boric acid/1.25 mM EDTA) and visualized by autoradiography.

RESULTS

First, sera from experimental and control mice were tested by indirect immunofluorescence on liver sections. Anti-nucleolar IgG autoantibodies were found in 11 of 11 $HgCl_2$-treated B10.S mice but in 0 of the 5 saline-injected control mice. The staining pattern is shown in Fig. 1. There is brilliant nucleolar staining, a faint staining of nuclear dots, and a dull nuclear staining showing mainly a peripheral pattern. In the 12th week after commencement of $HgCl_2$ injections, the titers of the anti-nucleolar antibodies ranged from 1:40,960 (mice nos. 1, 4, 8, and 11), >1:20,480 (nos. 5 and 7), and 1:5120 (nos. 2 and 6) down to 1:2560 (no. 3), 1:1280 (no. 9), and 1:640 (no. 10). The antibodies appear to recognize an evolutionarily highly conserved antigen, since they react with nucleoli from a wide variety of species in immunofluorescence, such as rat, kangaroo, mouse, and human (HeLa cells) (data not shown).

To identify the nucleolar antigen recognized by the $HgCl_2$-induced autoantibodies, immunoblots were prepared. When total proteins of nuclei from HeLa cells were used as antigens, the sera of 8 of 11 Hg-treated B10.S mice reacted with a single polypeptide with an apparent molecular mass of 36 kDa (Fig. 2, lanes 6–16). Sera with antibody titers of 1:1280 and below, as determined by immunofluorescence (mice nos. 3, 9, and 10), reacted only weakly in the immunoblot. Sera from B10.S mice that had been injected with saline only displayed no reaction (lanes 1–5), demonstrating that the autoantibody production against the 36-kDa protein is dependent on the exposure to $HgCl_2$.

Qualitatively similar results were obtained when total proteins from mouse Ehrlich ascites tumor cell nuclei were used as antigenic material for immunoblotting studies with the various mouse sera (data not shown), which demonstrates that true autoantibodies against the 36-kDa protein are being produced in B10.S mice after $HgCl_2$ treatment.

The correlation between the titer of anti-nucleolar autoantibodies in individual mouse sera and the intensity of the reaction of the respective sera with the 36-kDa protein on the immunoblots gave a first indication that the antigens recognized in both kinds of assay are identical. This notion was further substantiated by our finding that autoantibodies that had been affinity purified by elution from the 36-kDa protein after preparative immunoblots decorated the nucleoli specifically when examined by indirect immunofluorescence (Fig. 3).

The similarity in the apparent molecular mass of the proteins recognized by experimentally induced anti-nucleolar mouse autoantibodies (36 kDa) and the molecular

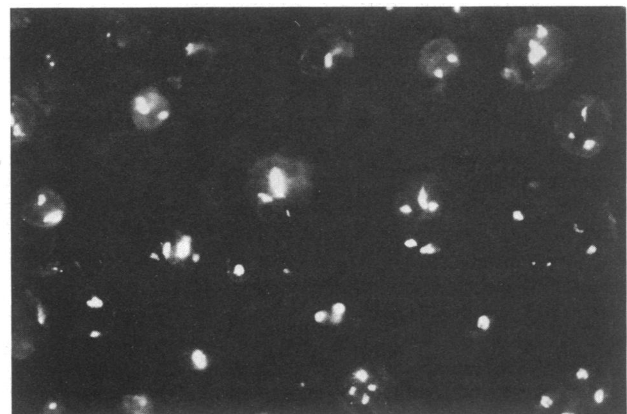

FIG. 1. Indirect immunofluorescence of $HgCl_2$-induced antiserum from a B10.S mouse on rabbit liver. The serum was taken at week 6 after the onset of $HgCl_2$ treatment; it was used at a dilution of 1:10.

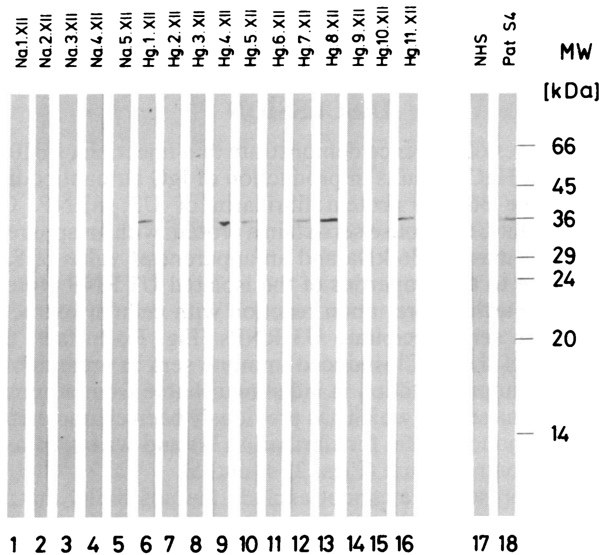

FIG. 2. Characterization by immunoblotting of the nuclear antigen recognized by $HgCl_2$-induced autoantibodies. Immobilized nuclear proteins from HeLa cells were probed with sera from B10.S mice treated with $HgCl_2$ for 12 weeks (lanes 6–16), with sera from B10.S mice treated with sodium chloride for 12 weeks (lanes 1–5), with serum from scleroderma patient S4 (lane 18), or with normal human serum (lane 17). All sera were diluted 1:200 in buffer A. Hg/m/n, serum sample of mouse no. *m* treated for *n* weeks with $HgCl_2$; Na/m/n, serum sample from a mouse treated with sodium chloride as a control.

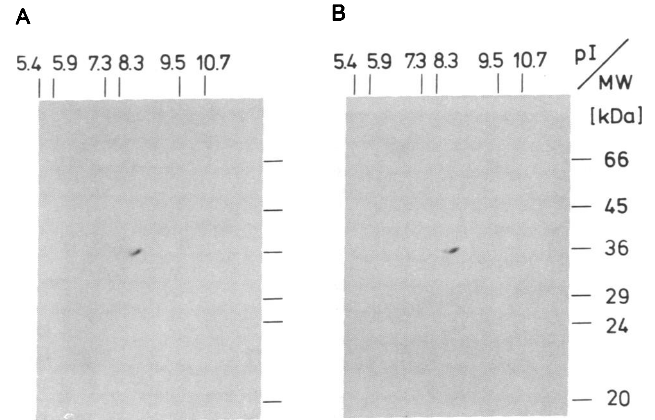

FIG. 4. Identification of the antigenic protein for the Hg-induced murine autoantibodies in a two-dimensional immunoblot of HeLa nuclear proteins. Total nuclear proteins from HeLa cells were separated by nonequilibrium pH gradient gel electrophoresis in the first dimension and by SDS/PAGE in the second dimension and were immobilized on nitrocellulose. (*A*) Immunoreactivity of the serum sample Hg/4/XII at a dilution of 1:200 in buffer A. (*B*) Immunoreactivity of scleroderma patient serum S4 at the same dilution.

mass of the U3 RNP protein (34–36 kDa), against which scleroderma sera react (see Introduction), suggested that $HgCl_2$ treatment of mice induces anti-U3 RNP autoantibody formation. This possibility was investigated in more detail. First it was shown that a serum from a scleroderma patient termed S4 and previously shown to react with the U3 RNP protein fibrillarin (31) decorated a single protein band of the same molecular mass as the mouse sera (Fig. 2).

We next compared the antigen reactivity of the sera from $HgCl_2$-treated mice and patient serum S4 in two-dimensional immunoblots. As demonstrated in Fig. 4, the two kinds of sera decorate indistinguishably the same protein with an apparent pI value of 8.6 and molecular mass 36 kDa (compare Fig. 4 *A* and *B* for the mouse and human sera, respectively). In fact, the double-label immunoblot, in which human and murine autoantibodies were allowed to react simultaneously with the two-dimensionally separated nuclear proteins, only decorated a single antigenic polypeptide with an apparent pI value of 8.6; this was irrespective of the order of addition of anti-mouse phosphatase and anti-human peroxidase antibodies used for displaying the autoantibody reaction (data not shown).

The physicochemical characteristics of the protein recognized by the mouse and human sera, as determined by our gel systems, closely agree with those described for the isolated U3 RNP protein fibrillarin (34 kDa, pI 8.5). Therefore, these data indicated that the experimentally induced autoantibodies were directed against the nucleolar protein fibrillarin. The above hypothesis was finally confirmed by radioimmunoprecipitation analysis. It has previously been shown that the anti-fibrillarin autoantibodies from scleroderma patients precipitate the nucleolar U3 RNP particle, demonstrating the association of this polypeptide with U3 RNA (11, 12). When cellular extracts from HeLa cells were allowed to react with high-titered anti-nucleolar antisera from $HgCl_2$-treated B10.S mice, U3 RNP was found in the immunoprecipitate (Fig. 5*A*, lanes 2 and 3), as would be anticipated for anti-fibrillarin autoantibodies.

Interestingly, various other RNAs were found in the immunoprecipitates obtained with the anti-nucleolar mouse antisera, in addition to U3 RNA (Fig. 5, lanes 2 and 3). This coprecipitation with U3 RNP of the other RNA molecules cannot be due to nonspecific interactions, since the mouse control sera do not display such immunoprecipitates (Fig. 5*A*, lanes 4 and 5). Furthermore, the same set of RNA molecules, in addition to U3 RNA, is also observed in the respective immunoprecipitate when anti-fibrillarin autoantibodies from scleroderma patient S4 reacted with HeLa cell extracts (lane 6). The presence of 5S and 5.8S rRNAs in the immunoprecipitates suggests that U3 RNP precipitated by the mouse and

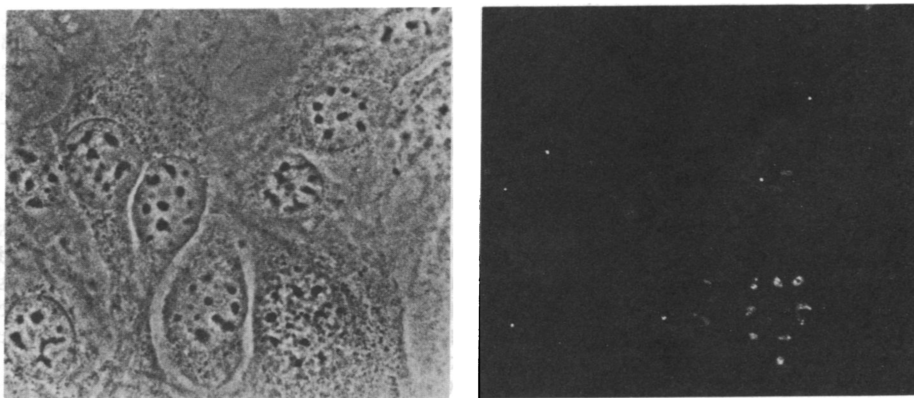

FIG. 3. Indirect cytoimmunofluorescence of autoantibodies eluted from immobilized 36-kDa protein. Cells were allowed to react with antibodies that were affinity purified from the immobilized 36-kDa protein; they were then labeled for indirect immunofluorescence and observed by epifluorescence optics.

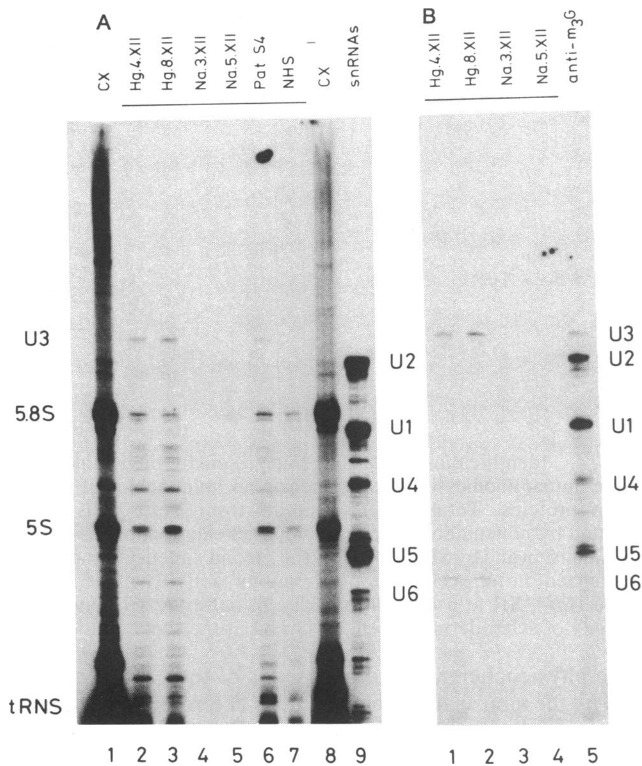

FIG. 5. PAGE of ^{32}P-labeled RNAs immunoprecipitated with autoantibodies from Hg-treated B10.S mice. (*A*) RNAs present in the immunoprecipitates of Hg-induced autoantibodies from B10.S mice (lanes 2 and 3), of sera from B10.S mice treated with NaCl (lanes 4 and 5), of serum from scleroderma patient S4 (lane 6), and of normal human serum (lane 7) were 3′-end-labeled and subjected to urea/PAGE. Lanes 1 and 8, 3′-end-labeled 4–8S RNAs of the low-speed HeLa cell extract (CX), which was used as antigen source; lane 9, 3′-end-labeled snRNAs obtained from a nuclear extract of HeLa cells by immune affinity chromatography with the anti-m^3G antibody (32). (*B*) RNAs present in the immunoprecipitates from a high-speed (lanes 1 and 3) and a low-speed (lanes 2 and 4) HeLa cell extract obtained with Hg-induced autoantibodies from B10.S mice (lanes 1 and 2) or with sera from B10.S mice mock-injected with NaCl (lanes 3 and 4) were reprecipitated by anti-m^3G IgG, 3′-end-labeled, and electrophoretically separated. Lane 5, 3′-end-labeled RNAs, which were precipitated from the total RNA of the high-speed extract by the anti-m^3G antibody.

human anti-fibrillarin autoantibodies is, at least in part, a component of preribosomal assembly intermediates, which is in accordance with previous observations (10). When the HeLa cell extract was freed from ribosomal particles by a high-speed centrifugation step at 120,000 × *g* for 2 hr (10), U3 RNP could still be precipitated efficiently by the mouse anti-fibrillarin autoantisera, indicating that a major part of U3 RNA is associated with the 36-kDa protein as free RNP particle in the cell extract (compare lanes 1 and 2 in Fig. 5*B*). This agrees with the findings of Parker and Steitz (12).

In the experiments shown in Fig. 5*B*, the immunoprecipitates obtained with the mouse sera were treated with phenol and the deproteinized RNAs were reprecipitated with anti-m^3G antibodies. It is therefore interesting that, in addition to U3 RNA, two further RNA species were found in the immunoprecipitates that migrate in a similar way to, but clearly distinct from, the nucleoplasmic snRNAs U4 and U6 (Fig. 5*B*). By the criterion of reactivity with anti-m^3G antibodies, these RNAs must contain 5′-terminal m^3G cap structures. While we cannot rigorously exclude the possibility that they are 5′-terminal degradation products from U3 RNA, the fact that they were observed reproducibly at similar abundance irrespective of the anti-36-kDa autoantisera used for the immunoprecipitation (i.e., various mouse and human autoantisera) does not support this possibility.

DISCUSSION

Several lines of evidence demonstrate that injection of B10.S mice with $HgCl_2$ results in production of IgG autoantibodies against the 36-kDa protein fibrillarin of U3 snRNP. On immunoblots, the mouse sera stain a protein with an apparent molecular mass of 36 kDa and an apparent pI value of 8.6, very close to the properties of the isolated U3 RNP protein (11, 12). Furthermore, upon reaction with cellular extracts, the mouse sera precipitate U3 RNPs (Fig. 5). In fact, the behavior of the $HgCl_2$-induced immune sera in immunoblot and immunoprecipitation is indistinguishable from a human scleroderma serum, which has previously been characterized as being monospecific for fibrillarin (31) and was used as a positive control serum (Figs. 2, 4, and 5).

At the present, we cannot exclude the possibility that, in addition to anti-nucleolar autoantibodies, $HgCl_2$-treated B10.S mice also produce other autoantibodies. Clearly, however, anti-U3 RNP antibodies dominate the autoimmune response of these mice. This is indicated by our finding that the strength of the nucleolar immunofluorescence of individual mouse sera closely correlated with the intensity by which they stained the 36-kDa protein in the immunoblot. Furthermore, mouse autoantibodies that had been eluted from the 36-kDa protein on preparative immunoblots specifically stained the nucleoli in the immunofluorescence assay (Fig. 3). How can we explain this surprisingly selective autoantibody production in B10.S mice by $HgCl_2$? At least three mechanisms may be considered, which are not mutually exclusive.

(*i*) The 36-kDa protein may specifically be modified by $HgCl_2$ and thus rendered "foreign" to T lymphocytes.

(*ii*) $HgCl_2$ is known to exert a direct stimulating effect on T helper cells, the mechanism of which is not understood (33–35). The activation of T helper cells seems to activate B cells to the increased synthesis of IgG and IgE noted in $HgCl_2$-treated mice (36). The activation of B cells, in turn, might include and selectively favor those B cells that recognize the 36-kDa protein. A similar mechanism has already been discussed for the preferential production of autoantibodies against DNA and histones, which is observed in mice during chronic graft versus host disease (37).

(*iii*) $HgCl_2$ might directly modify major histocompatibility complex class II molecules of B10.S mice. This possibility is consistent with the fact that $HgCl_2$ can be a contact sensitizer (38, 39), implying that it creates a specific epitope seen by T cells. This mechanism, too, would result in a stimulation of T helper cells, which in turn might preferentially activate fibrillarin-specific B cell, as described in *ii*. In the latter two cases (*ii* and *iii*), helper T cells would cooperate with the autoreactive B cells via a "noncognate" mechanism, and we still have to settle the question of which structural features render the unmodified 36-kDa protein particularly active for autoimmunization, a question that must be answered to explain the preferential triggering of fibrillarin-specific B cells. In chronic graft versus host disease, both the structure and the available concentration of self-antigen appear to account for the preferential triggering of only certain autoreactive B cells by noncognate T-cell–B-cell collaboration (37, 40).

Recently, Hultman and Eneström (41) reported that SJL (H-2^s) mice, too, produce anti-nucleolar IgG autoantibodies upon treatment with $HgCl_2$. Furthermore, they provided experimental evidence that these anti-nucleolar antibodies might be pathogenically active. It is likely, therefore, that the high titers of IgG autoantibodies to U3 snRNP reported in the present paper also are pathogenic. Consistent with this view, $HgCl_2$-treated B10.S mice show granular IgG deposits in the

mesangium, the walls of their glomerular capillaries, and other blood vessel walls (42).

It is striking that $HgCl_2$ induces autoantibody production against the same nucleolar antigen against which autoantibodies are being produced spontaneously by patients suffering from idiopathic scleroderma. Both the murine and human autoantibodies are directed against evolutionarily highly conserved regions on the fibrillarin protein. Thus, not only do scleroderma patient anti-U3 RNP antisera react with the respective antigen from species as diverse as vertebrates (13), plants (43), and yeast (44), but the $HgCl_2$-induced mouse anti-U3 RNP antibodies react with all these as well (unpublished observations). A comparative analysis of the autoimmunizing B-cell epitopes and of the T-cell epitopes in the two systems will be necessary to see whether environmental toxins, such as mercurials, may be a cause of the autoantibody production seen in scleroderma patients.

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