

Induction of Lupus-associated Autoantibodies in BALB/c Mice by Intraperitoneal Injection of Pristane

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Summary

Intraperitoneal injection of pristane (2,6,10,14 tetramethylpentadecane) is a standard technique for obtaining monoclonal antibody-enriched ascitic fluid. However, pristane also induces plasmacytomas and an erosive arthritis resembling rheumatoid arthritis in BALB/c mice, probably as a consequence of enhanced interleukin 6 production. We report here that the production of autoantibodies characteristic of systemic lupus erythematosus (SLE) is a further consequence of injecting pristane in BALB/c mice. Anti-Su antibodies appeared as early as 1–2 mo after a single injection of 0.5 ml pristane, followed by anti-U1RNP and anti-Sm antibodies after 2–4 mo. Within 6 mo of pristane injection, 9 of 11 BALB/c mice had developed anti-Su, anti-U1RNP, anti-U2RNP, anti-Sm, and possibly anti-U5RNP antibodies. Autoantibodies were not produced by 20 BALB/c mice of the same age and sex that were not injected with pristane. Thus, autoantibodies characteristic of lupus were induced in mice that are not usually considered to be genetically susceptible to the disease. The induction of autoantibodies associated with SLE by pristane may be relevant to understanding the role of abnormal cytokine production in autoantibody production and the pathogenesis of autoimmune disease. Furthermore, the induction of high titer autoantibodies by pristane dictates caution in the use of ascitic fluid as a source of monoclonal antibodies, since the polyclonal autoantibodies induced by pristane may copurify with the monoclonal antibody secreted by an injected hybridoma.

Intraperitoneal administration of pristane (2,6,10,14 tetramethylpentadecane) before the injection of hybridoma cells is a standard technique for obtaining ascitic fluid containing a high concentration of mAbs. In addition to its effects on hybridoma cell growth, pristane-induced alterations in cytokine production have been implicated in the pathogenesis of plasmacytomas (1–3) and erosive arthritis resembling rheumatoid arthritis (4, 5). While characterizing a slowly growing murine hybridoma secreting an IgM mAb, we observed that ascitic fluid from several pristane-primed BALB/c mice injected with hybridoma cells contained polyclonal IgG autoantibodies to Su, U1RNP, U2RNP, and/or Sm. Further investigation revealed that the autoantibodies were a consequence of pristane priming itself, and were unrelated to the hybridoma cells or their secreted monoclonal IgM. Thus, intraperitoneal injection of pristane induced lupus-like autoimmunity in a strain of mouse not usually thought to be prone to autoimmune disease.

Materials and Methods

Cell Lines. The K562 (human erythroleukemia) and L929 (murine fibroblast) cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and maintained in

RPMI 1640 or MEM, respectively, supplemented with 9% FCS, L-glutamine, and penicillin/streptomycin.

Sera and mAbs. Prototype human autoimmune sera containing anti-Su, anti-U1RNP, anti-Sm, or other specificities, were reported previously (6–8). Additional sera with anti-U1RNP/Sm antibodies were obtained from patients with systemic lupus erythematosus (SLE) followed at the University of North Carolina Hospitals (Chapel Hill, NC) or the Keio University Hospital (Tokyo, Japan). Murine mAbs 2.73 (anti-U1-70K) (9), and 9A9 (anti-U1-A and U2-B'') (10) were provided by Dr. Yoshihiko Takeda (Medical College of Georgia, Augusta, GA) and Dr. W.J. van Venrooij (University of Nijmegen, The Netherlands), respectively. mAbs Y2 (anti-Sm B'/B and D) (11), 22G12 (anti-Sm B'/B) (12), and 2G7 (anti-Sm-D) (13) were provided by Dr. Robert A. Eisenberg (University of North Carolina).

Pristane Priming. 6–8-wk-old female BALB/c ByJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained at our animal facility. Eleven mice, ages 4–5 mo, received a single intraperitoneal injection of 0.5 ml of pristane (Sigma Chemical Co., St. Louis, MO). Sera were collected every 4 wk from the tail vein. 20 age- and sex-matched BALB/c ByJ mice that were not injected with pristane served as controls.

Immunoprecipitation. Immunoprecipitation using cell extract from K562 or L929 cells was performed as described previously (7, 8). Briefly, the cells were labeled for 14 h with [³⁵S]methionine/cysteine (25 μCi/ml), lysed in 0.5 M NaCl NET/NP-40 buffer

(500 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl, pH 7.5, 0.3% NP-40), and the extract cleared by centrifugation. Labeled cell extract derived from 1.5×10^6 cells was incubated for 90 min with 4 μ l of mouse serum plus 12 μ l of rabbit anti-mouse IgG (1 mg/ml), or with 5 μ l of human serum, absorbed onto 15 μ l of packed protein A-Sepharose beads. The beads were washed three times with 0.5 M NaCl NET/NP-40, and once with NET buffer (150 mM NaCl, 2 mM EDTA, 50 mM Tris-HCl, pH 7.5), and immunoprecipitated proteins were analyzed by SDS-PAGE.

Pulse Labeling and Immunoprecipitation. K562 cells were pulse-labeled for 6 min with [35 S]methionine/cysteine as described (7), and extract derived from 3×10^6 cells was immunoprecipitated as above with mAbs 2.73, 9A9, Y2, 22G12, or 2G7, human autoimmune sera, or 4 μ l of sera from mice injected with pristane. Immunoprecipitates were washed three times with mixed micelle buffer (MMB, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.25 M sucrose, 2.5% Triton X, 0.5% SDS) and once with NET, and analyzed by SDS-PAGE.

Immunoblotting of Sera with Purified U1RNP Antigen. U1RNP antigen was immunoaffinity purified from K562 cell extract as described (14), fractionated on a 12.5% SDS polyacrylamide gel, and transferred to a nitrocellulose membrane. Strips cut from the membrane were incubated for 90 min with 1:250 diluted ascitic fluid, 1:3 diluted culture supernatant of mAbs 2.73, 9A9, Y2, or 2G7, or 1:250 diluted human or murine serum. After washing, the strips were incubated an additional 90 min with alkaline phosphatase-conjugated goat antibodies specific for either murine or human IgG (Fisher Scientific, Norcross, GA) and developed as described previously (6).

Results

Induction of Anti-Su Antibodies by Pristane. 5 mo after receiving a single intraperitoneal injection of pristane, sera from 5/11 BALB/c ByJ mice immunoprecipitated 100/102- and 200-kD polypeptides from extracts of 35 S-labeled human K562 erythroleukemia cells (Fig. 1, lanes 1, 2, 8, 10, and 11). As shown in Fig. 1, the 100/102- and 200-kD proteins comigrated with the proteins immunoprecipitated by anti-Su prototype serum (6). The identity of the polypeptides with Su was confirmed by preclearing experiments (not shown). The sera also formed a line of identity with human and murine anti-Su reference sera in Ouchterlony double immunodiffusion (not shown), and immunoprecipitated similar polypeptides from murine L-929 cell extract. Sera from control mice were negative (Fig. 1, lane C). Autoantibodies to Su are strongly associated with the diagnosis of lupus in both humans and MRL/lpr mice (15), suggesting that pristane induced the production of autoantibodies characteristic of SLE in BALB/c mice. That possibility was further supported by the detection of serum autoantibodies to the components of U1, U2, and U5 small nuclear ribonucleoproteins (snRNPs).

Induction of Autoantibodies to U1, U2, and U5 snRNPs by Pristane. The U series of snRNPs carry the shared "Sm" proteins B'/B, D, E, F, and G that form a 6S core precursor particle common to U1, U2, U4-6, and U5 snRNPs (7, 8, 16), as well as other proteins unique to individual snRNPs. The unique proteins of U1 snRNPs include U1-70K, U1-A, and U1-C (17). U2 snRNPs carry the unique proteins U2-A' and U2-B'' (17, 18), and U5 snRNPs carry unique proteins

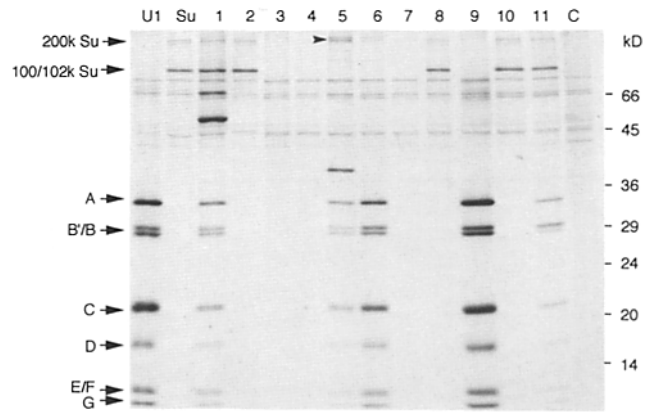


Figure 1. SDS-PAGE of 35 S-labeled proteins immunoprecipitated by sera of pristane-primed mice. Extract from K562 cells labeled 14 h with [35 S]methionine/cysteine was immunoprecipitated with 4 μ l of mouse sera obtained 5 mo after pristane injection, followed by washing with 0.5 M NaCl NET/NP-40 as described in Materials and Methods. Immunoprecipitates were analyzed on a 12.5% SDS gel and autoradiographed. U1 and Su lanes show immunoprecipitation with prototype anti-U1RNP and anti-Su sera, respectively. Lanes 1–11 show immunoprecipitation with sera from 11 pristane-primed BALB/c mice. Lane C shows immunoprecipitation with serum from a mouse that was not injected with pristane. Positions of the U1 snRNP-specific A and C proteins, the Sm proteins B'/B, D, E/F, and G, and the 200- and 100/102-kD Su-related proteins are indicated. Positions of molecular weight standards in kilodaltons are shown on the right.

of 200 and 205 kD (doublet) (19). Antibodies specific for the unique proteins of U1, U2, and U5 snRNPs will be referred to as anti-U1RNP, anti-U2RNP, and anti-U5RNP, respectively, whereas those recognizing the shared components B'/B, D, E, F, or G will be referred to as anti-Sm.

Five sera from mice injected with pristane immunoprecipitated the polypeptides A, B'/B, C, D, E, F, and G typical of U1 snRNPs from extracts of cells labeled for 14 h (Fig. 1, lanes 1, 5, 6, 9, and 11). Like anti-Su, autoantibodies to U1 snRNPs are strongly associated with SLE in humans (11, 20) as well as mice (21–23). Two sera also immunoprecipitated the 200/205-kD protein components of U5 snRNPs (19) at 5 mo (Fig. 1, lanes 5 and 6, *arrowhead*), and three additional mice developed these antibodies at 6 mo (not shown).

The fine specificities of the anti-snRNP antibodies in sera from pristane primed mice were investigated by pulse labeling and immunoprecipitation (Fig. 2 A). This technique permits evaluation of autoantibody binding to newly synthesized snRNP proteins before the assembly of mature snRNP particles (7, 8). As expected, murine mAbs immunoprecipitated different subsets of snRNP polypeptides from extracts of 6-min pulse-labeled K562 cells. Although mAb 2.73 (Fig. 2 A, lane 1) is specific for the U1-70K protein (9), it immunoprecipitated U1-A and an unidentified 28-kD protein after pulse labeling. The U1-70K polypeptide is radiolabeled poorly (7), and was therefore not well visualized. In accordance with its specificity for U1-A and U2-B'' (10), mAb 9A9 immunoprecipitated both of these proteins (U2-B'' has similar mobility to Sm-B', see Fig. 2, lane 2, *arrowhead*), but not U1-C

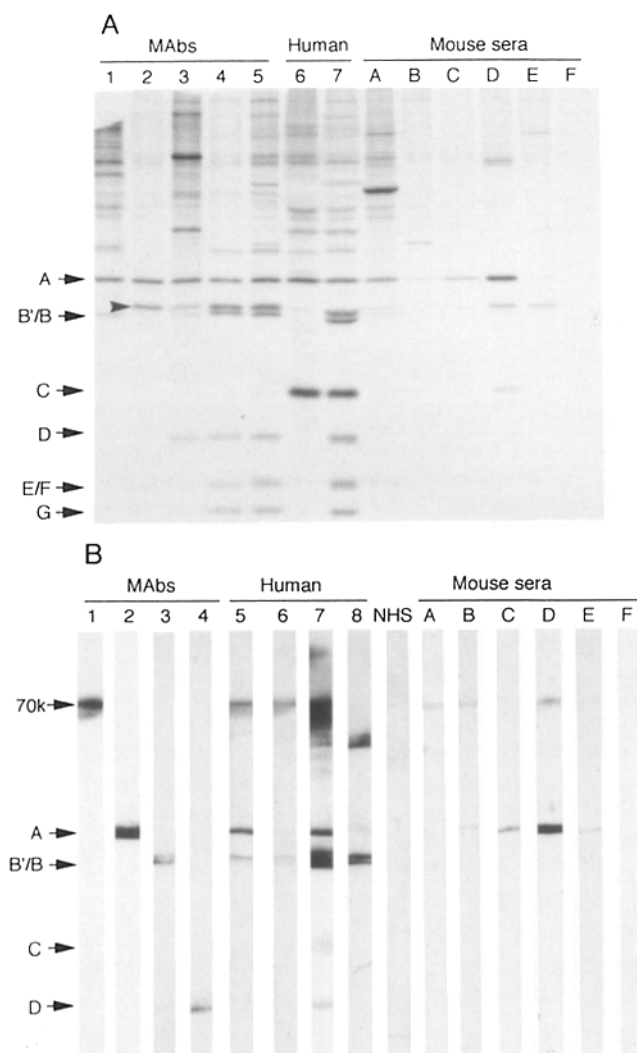


Figure 2. Specificity of pristane-induced anti-U1RNP antibodies. (A) Immunoprecipitation of 6-min pulse-labeled cell extract. K562 cells were pulse-labeled with [³⁵S]methionine/cysteine for 6 min, and extract was immunoprecipitated with mAbs 2.73, 9A9, Y2, 22G12, and 2G7, human autoimmune sera, or sera from mice injected with pristane. Immunoprecipitates were washed with MMB and analyzed on a 12.5% SDS-polyacrylamide gel. Immunoprecipitation of pulse-labeled K562 cell extract with mAbs specific for snRNP components is shown in lanes 1–5. Lane 1, mAb 2.73 (anti-U1-70K); lane 2, mAb 9A9 (reactive with U1-A and U2-B'' proteins); lane 3, mAb 22G12 (anti-Sm B'/B); lane 4, mAb Y2 (anti-Sm B'/B and D proteins); lane 5, mAb 2G7 (anti-Sm-D). Immunoprecipitation of pulse-labeled cell extract with human autoimmune sera is shown in lanes 6 and 7. Lane 6, anti-U1RNP positive human serum; lane 7, anti-U1RNP plus anti-Sm positive human serum. Immunoprecipitation using anti-U1RNP positive sera from mice injected 5 mo previously with pristane is shown in lanes A–E. Lane F shows immunoprecipitation with serum from mouse A before pristane was injected. (B) Western blot analysis of sera using purified U1RNP antigen. U1RNP antigen was immunoaffinity purified from K562 cell extract, fractionated on a 12.5% SDS polyacrylamide gel, and transferred to a nitrocellulose membrane. Strips cut from the membrane were incubated with diluted mAbs or human or murine sera as described in Materials and Methods, followed by alkaline phosphatase-conjugated goat antibodies specific for either murine or human IgG. Lanes 1–4, mAbs 2.73 (anti-U1-70K), 9A9 (anti-U1-A and U2-B''), Y2 (anti-Sm B'/B and D), and 2G7 (anti-Sm-D), respectively. Lanes 5 and 6, human autoimmune sera with anti-U1RNP antibodies; lanes 7 and 8, human anti-Sm/U1RNP positive sera. NHS, normal human serum.

or the Sm proteins B'/B, D, E, F, or G. The latter were immunoprecipitated, as expected, by the anti-Sm mAbs 22G12 (anti-Sm B'/B [12], Fig. 2, lane 3), Y2 (anti-Sm B'/B and D [11], Fig. 2, lane 4), and 2G7 (anti-Sm D [13], Fig. 2 lane 5).

As shown previously (7), human autoimmune sera containing anti-U1RNP antibodies immunoprecipitated exclusively the U1-A and U1-C polypeptides, whereas sera containing anti-Sm antibodies immunoprecipitated the Sm B'/B, D, E, F, and G proteins as well as U1-A and U1-C (Fig. 2 A, lanes 6 and 7, respectively). All sera from pristane primed mice that immunoprecipitated proteins A–G from 14 h labeled cell extract immunoprecipitated U1-A from pulse labeled cell extract (Fig. 2 A, lanes A–E), whereas serum from a control BALB/c mouse not injected with pristane was negative (Fig. 2 A, lane F). One of the sera from a pristane primed mouse also immunoprecipitated U1-C (Fig. 2 A, lane D), and two immunoprecipitated U2-B'' (Fig. 2 A, compare lanes D and E with lane 2). None of the mouse sera immunoprecipitated the Sm proteins D, E, F or G, and the single serum that immunoprecipitated B'/B did so weakly (Fig. 2 A, lane A). The pattern displayed by sera from pristane primed mice was similar to that of anti-U1RNP mAbs 2.73 and 9A9 or human autoimmune sera containing anti-U1RNP antibodies, suggesting that U1RNP, and not the Sm core proteins, was the major target of anti-snRNP autoantibodies in these sera. This interpretation was confirmed by Western blotting using affinity U1 snRNPs (Fig. 2 B).

In agreement with the pulse labeling experiments, the sera of mice injected with pristane showed reactivity by Western blotting with U1-A (4/5 sera strongly, and 1/5 weakly positive). Reactivity with U1-70K (3/5 sera strongly, and 1/5 weakly positive) was also apparent, whereas reactivity with Sm-B'/B was weak or absent (Fig. 2 B, lanes A–E). None of the sera was reactive with Sm-D. IgG1, IgG2a, IgG2b, and IgG3 autoantibodies were all detected by Western blot using isotype-specific second antibodies (not shown). In contrast to the BALB/c mouse sera, human autoimmune sera containing anti-Sm antibodies exhibited strong reactivity with B'/B (Fig. 2 B, lanes 7–8) and D (Fig. 2 B, lane 7), whereas normal human serum was negative. As expected, murine mAb 2.73 was reactive on blots with U1-70K (Fig. 2 B, lane 1), and 9A9 reacted with U1-A (Fig. 2 B, lane 2). Since affinity-purified U1 snRNPs were used as antigen, reactivity of 9A9 with U2-B'' was not apparent. mAb Y2 showed strong reactivity with Sm-B'/B and weaker binding to Sm-D (Fig. 2 B, lane 3), whereas 2G7 showed reactivity with D, but not B'/B. Taken together, the pulse labeling and Western blot studies indicated that sera obtained from BALB/c mice 5 mo after a single pristane injection contained autoantibodies specific for components of U1 snRNPs (primarily the U1-70K and U1-A proteins) and U2 snRNPs (the U2-B'' protein).

Time Course of Autoantibody Production. Although arthritis

Lanes A–E show immunoblot analysis of mouse sera obtained 5 mo after pristane injection. Lane F shows immunoblot using serum from mouse A before pristane was injected.

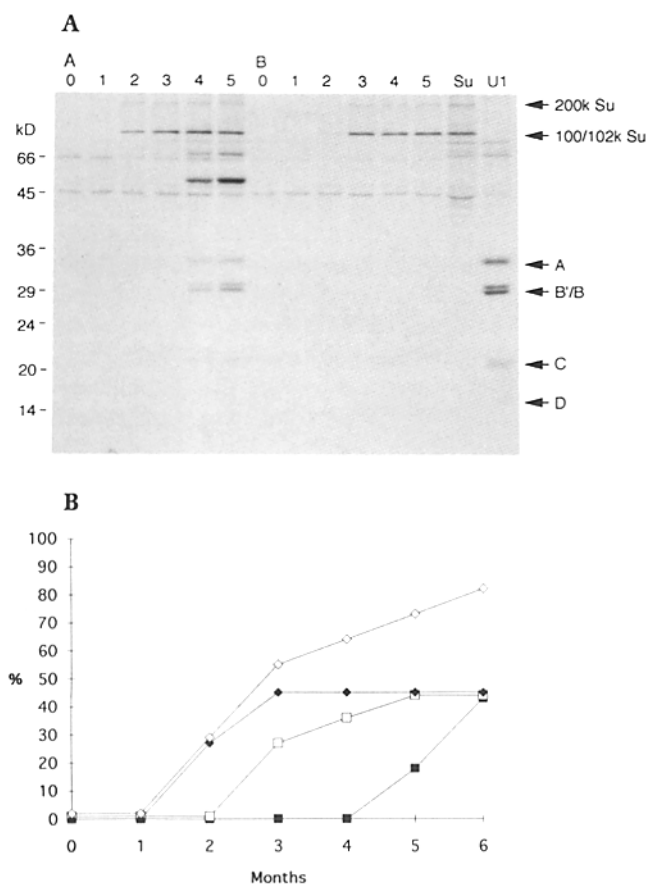


Figure 3. (A) Immunoprecipitation with serial sera from pristane-primed mice. Serial serum samples (4 μ l) obtained from two mice (mice A and B, respectively) before injecting pristane, and at 1, 2, 3, 4, and 5 mo after pristane was injected, were tested by immunoprecipitating 14 h radiolabeled K562 cell extract. Positions of 100/102 and 200 kD Su antigens and U1-A, U1-C, and Sm proteins B'/B and D are indicated. Positions of molecular weight standards are shown on the left. (B) Time of onset of anti-Su, anti-U1RNP, and anti-U5RNP antibodies. Sera from 11 pristane-primed BALB/c mice were tested at monthly intervals for anti-Su, anti-U1RNP, and anti-U5RNP antibodies by immunoprecipitation. The percentage of sera positive for anti-Su (solid diamonds), anti-U1RNP (open squares), U5RNP (solid squares), or any of the three (open diamonds), over time is shown.

occurs 4–6 mo after the injection of pristane (4, 5), examination of the ascitic fluid of mice injected with hybridoma cells suggested that autoantibody production might commence earlier than that. Thus, the time course of autoantibody production by pristane primed mice was investigated. As shown in Fig. 3, anti-100/102 and 200 kD Su antibodies generally appeared between 1 and 2 mo after pristane priming (Fig. 3 A–B, solid diamonds), whereas anti-U1RNP antibodies appeared somewhat later, between 2 and 3 mo after priming (Fig. 3 B, open squares). Autoantibodies that immunoprecipitated the characteristic 200/205-kD doublet of U5 snRNPs (e.g., Fig. 1, lane 5), appeared still later, after 4–6 mo (Fig. 3 B, solid squares). By 6 mo, 9/11 pristane primed mice (82%) had developed anti-Su, anti-U1RNP, and/or anti-U5RNP autoantibodies (5/11 anti-Su, 5/11 anti-U1RNP, and 5/11 anti-U5RNP) (Fig. 3 B, open diamonds). The U snRNP and Su antigens from human K562 (Figs. 1–3) and murine L-929

(not shown) cell extracts were immunoprecipitated comparably, indicating that the sera contained true autoantibodies to U snRNPs and Su.

Discussion

The induction of autoantibodies to Su, U1-A, U1-70K, U2-B'', and Sm-B'/B, all of which are associated with SLE, in BALB/c mice by pristane has several implications. First, the "spontaneous" production of these autoantibodies is not limited to autoimmune strains of mice, suggesting that environmental factors may play a role in initiating anti-Su and anti-snRNP antibodies. It is of interest that, as in pristane-primed BALB/c mice, anti-U1-A and anti-U1-70K autoantibodies are detected in the sera of MRL/lpr mice with spontaneous autoimmune disease before other anti-snRNP antibodies appear (22). In view of the sequence similarity and immunological crossreactivity of U1-70K and the retroviral p30^{gag} antigen (24), it is possible that reactivation of an endogenous retrovirus as a consequence of pristane injection (25) was responsible for the early generation of anti-U1-70K autoantibodies in the pristane-primed mice. However, further studies will be needed to evaluate that possibility. The later development of anti-Sm B'/B and anti-U5RNP antibodies in some of the pristane-primed mice may reflect intermolecular/intrastructural diversification of the immune response to these snRNPs, as suggested previously (23). Thus, the production of autoantibodies after injecting BALB/c mice with pristane resembles spontaneous autoantibody production in MRL/lpr mice in several respects. We have observed recently that BALB/c mice develop significant proteinuria within several months after pristane injection, suggesting that similarity between the pristane model and spontaneous autoimmunity in MRL/lpr mice includes the development of glomerulonephritis.

In addition to their relevance to the pathogenesis of autoimmunity, the present observations have broader significance relating to the commonly used technique of producing mAbs in pristane-primed BALB/c mice (26). In view of the rapid appearance of anti-Su antibodies after pristane injection, mAbs isolated from the ascitic fluid of pristane-primed mice injected with hybridoma cells may be contaminated by considerable amounts of polyclonal IgG autoantibodies. The possibility of autoantibody contamination of ascitic fluid will need to be taken into account, especially in the case of slowly growing hybridomas. We found that ascitic fluid from mice injected with a slowly growing IgM secreting hybridoma contained anti-Su antibodies of the IgG1, IgG2a, IgG2b, and IgG3 isotypes, and evidence of similar contamination of other mAbs prepared from ascitic fluid at other institutions has been found (data not shown). Purification of mAbs from culture supernatants should be considered to avoid this problem.

The practice of growing hybridomas as ascites tumors in pristane-primed mice (26) is based on the observation that pristane, a common component of many mineral oils, is plasmacytomagenic in susceptible strains of mice and enhances the ease of transplanting these tumors (1). Pristane is a peri-

toneal irritant that causes a chronic granulomatous inflammatory response characterized by high levels of IL-6 production (27). Plasmacytomas arise in the oil-induced granulomas after several months, and their frequency is increased by multiple injections of pristane. IL-6 produced by the macrophages and other cells within the oil-induced granulomas is an essential growth factor for plasmacytomas and is thought to be instrumental to the pathogenesis of these tumors (2, 27, 28). In addition to enhancing the growth of plasmacytomas and the production of mAb enriched ascitic fluid, pristane has other effects, including the reactivation of endogenous retroviruses (25) and the development of erosive arthritis resembling rheumatoid arthritis, accompanied by rheumatoid factor and autoantibodies to type II collagen (4, 5). These effects are not seen until 4–6 mo after pristane injection, and generally require multiple pristane injections. Unlike the development of plasmacytomas and arthritis, and the release of infectious murine leukemia viruses (25), the production of autoantibodies typical of SLE was an early effect of pristane, seen as early as 1–2 mo after a single dose.

Although the mechanism of autoantibody production in pristane-primed BALB/c mice is uncertain, overproduction of IL-6, which has been reported in these mice (27), is associated with both autoantibody production and autoimmune disease (29). IL-6 transgenic mice develop IgG1 plasmacytosis, hypergammaglobulinemia, and mesangioproliferative glomerulonephritis (28). Moreover, SLE B cells secrete high levels of IL-6 spontaneously (30), and tumors that overproduce IL-6, such as atrial myxomas, are associated with autoimmune phenomena (29). Thus, there is considerable evidence for a link between IL-6 overproduction and autoimmunity. Although it remains to be shown that IL-6 is involved in the pathogenesis of pristane-induced autoantibodies, the present study indicates that “normal” mice can produce lupus autoantibodies when exposed to a substance known to enhance the production of IL-6. Further investigation of the pathogenesis of autoantibodies typical of lupus and autoimmune disease (e.g., nephritis) in pristane primed mice may provide new insight into the causes of SLE.

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