

Interleukin 6 Dependence of Anti-DNA Antibody Production: Evidence for Two Pathways of Autoantibody Formation in Pristane-induced Lupus

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Summary

Pristane induces a lupus-like syndrome in nonautoimmune mice characterized by the development of glomerulonephritis and lupus-associated autoantibodies. This is accompanied by overproduction of interleukin (IL)-6, a cytokine linked with autoimmune phenomena. The goal of this study was to evaluate the role of IL-6 in autoantibody production in pristane-induced lupus. BALB/cAn IL-6-deficient (-/-) and -intact (+/+) mice were treated with pristane or phosphate-buffered saline, and autoantibody production was evaluated. Pristane induced high levels of immunoglobulin (Ig)G anti-single-stranded DNA, -double-stranded (ds)DNA, and -chromatin antibodies in IL-6^{+/+}, but not IL-6^{-/-} mice by enzyme-linked immunosorbent assay. High titer IgG anti-dsDNA antibodies also were detected in sera from +/+, but not -/-, mice by *Crithidia luciliae* kinetoplast staining. The onset of IgG anti-dsDNA antibody production in +/+ mice occurred >5 mo after pristane treatment, well after the onset of nephritis, suggesting that these antibodies are not directly responsible for inducing renal disease. In contrast to anti-DNA, the frequencies of anti-nRNP/Sm and anti-Su antibodies were similar in pristane-treated IL-6^{-/-} and IL-6^{+/+} mice. However, levels were higher in the +/+ group. These results suggest that IgG anti-DNA and chromatin antibodies in pristane-treated mice are strictly IL-6 dependent, whereas induction of anti-nRNP/Sm and Su autoantibodies is IL-6 independent. The IL-6 dependence of anti-DNA, but not anti-nRNP/Sm, may have implications for understanding the patterns of autoantibody production in lupus. Anti-DNA antibodies are produced transiently, mainly during periods of disease activity, whereas anti-nRNP/Sm antibody levels are relatively insensitive to disease activity. This may reflect the differential IL-6 dependence of the two responses.

Key words: systemic lupus erythematosus • pristane • antinuclear antibodies • interleukin 6 • anti-DNA antibodies

BALB/c, SJL/J, and C57BL/6 mice injected intraperitoneally with pristane develop lupus-specific autoantibodies, including anti-nRNP/Sm and ribosomal P, as well as the less disease-specific autoantibodies anti-Su, histone, and single-stranded (ss)DNA (1–3). They also develop severe immune complex-mediated glomerulonephritis starting 4–6 mo after pristane treatment (2, 3). Anti-double-stranded (ds)DNA antibodies, which are implicated in the pathogenesis of lupus nephritis (4, 5), have so far not been observed, raising questions about the role of anti-dsDNA antibodies in the development of nephritis in these mice.

IL-6 production by an expanded macrophage compartment is thought to be instrumental in the pathogenesis of

anti-DNA antibodies and nephritis in (NZB/W)F1 mice (6, 7). In light of these findings and the fact that large amounts of IL-6 are produced upon uptake of pristane by murine peritoneal macrophages (8, 9), we investigated the role of this cytokine in autoantibody production in pristane-induced lupus. Our data indicate that production of IgG anti-ssDNA, antichromatin and anti-dsDNA antibodies in pristane-treated mice requires IL-6, suggesting that anti-DNA antibody production in lupus is a marker of IL-6 overproduction. Unexpectedly, the frequencies of anti-nRNP/Sm and Su autoantibodies were similar in IL-6 deficient versus intact mice, suggesting that pristane induces autoantibody production by both IL-6-independent and -dependent pathways.

Materials and Methods

Mice. Mice were generated by five backcrosses of B6/129SV IL-6^{-/-} with BALB/cAn. Mice heterozygous for the IL-6 mutation were intercrossed to create IL-6^{-/-} and IL-6^{+/+} mice and were genotyped by Southern blotting (10). Female BALB/cAn IL-6^{-/-} ($n = 28$) and IL-6^{+/+} ($n = 26$) mice, age 10–12 wk, were injected once intraperitoneally with 0.5 ml of pristane (2,6,10,14-tetramethylpentadecane; Sigma Chemical Co., St. Louis, MO) (1). Control mice (IL-6^{-/-}, $n = 10$ and IL-6^{+/+}, $n = 10$) received 0.5 ml of PBS intraperitoneally. Sera were collected from the tail vein before treatment, at 3 wk, and at 3, 5, and 8 mo after treatment. Mice were housed under specific pathogen-free conditions.

Immunoprecipitation. Analysis of autoantibody specificities by immunoprecipitation was carried out as described previously (1).

ELISAs. Antigen capture ELISAs for anti-nRNP/Sm and anti-Su antibodies were performed as described, using mouse sera at a dilution of 1:500 and goat anti-mouse IgG second antibodies (γ chain-specific, from Southern Biotechnology Associates, Birmingham, AL) (11, 12). Anti-ssDNA ELISA was performed as previously described (13), and data were analyzed using the Mann Whitney test.

Anti-dsDNA antibody ELISA was carried out as previously described with minor modifications (14). In brief, calf thymus DNA (Sigma Chemical Co.) was extracted with phenol/chloroform/isoamyl alcohol and digested with S1 nuclease (GIBCO BRL, Gaithersburg, MD). Dynex polystyrene microtiter plates (Dynastar, Dynatech, Chantilly, VA) were coated with 100 μ l/well of 5 μ g/ml DNA in SSC, pH 8.0, and incubated at 37°C for 12 h. Sera were tested at a 1:160 dilution followed by alkaline phosphatase-conjugated goat anti-mouse IgG antibodies. Substrate was added, and $A_{405\text{ nm}}$ was determined. Absorbances from blank wells (no serum added) were subtracted.

IgG antichromatin antibodies were measured as previously described (15). In brief, polyvinyl chloride microtiter plates were coated with 10 μ g/ml chicken erythrocyte chromatin in borate-buffered saline. Sera were tested at a 1:500 dilution, followed by biotinylated goat anti-mouse IgG (pFc'-specific, 1:4,000 dilution, from Dr. P.L. Cohen, University of North Carolina, Division of Rheumatology and Immunology), avidin-conjugated alkaline phosphatase (Zymed Labs, South San Francisco, CA), and *p*-nitrophenyl phosphate substrate. The $A_{405\text{ nm}}$ of a 1:1,000 dilution of a high titer MRL/*lpr* reference serum was assigned a value of 2,048 units, and a 2¹¹-fold dilution of this standard a value of 2 units (15). IgG antichromatin activity in units for each sample was determined as in reference 12.

Fluorescence Assay for Anti-dsDNA Antibodies. Anti-dsDNA antibodies were measured by the *Crithidia luciliae* kinetoplast staining assay (16) at a serum dilution of 1:20 according to the manufacturer's instructions (The Binding Site, Birmingham, UK). Second

antibody was FITC-conjugated goat anti-mouse IgG (1:40 dilution; Southern Biotechnology Associates). All positive sera were titered (1:40, 1:80, 1:160, 1:320, and 1:640 dilutions).

Results and Discussion

IL-6 has been implicated in both anti-DNA antibody production and the pathogenesis of nephritis in (NZB/W)F1 mice (6, 7), and in the development of autoantibodies in patients with cardiac myxomas (17, 18). It also is essential for the growth of plasmacytomas in pristane-treated mice (8, 19–21). This study was undertaken to evaluate the role of this cytokine in the induction of autoantibodies by pristane in BALB/c mice.

Anti-DNA Antibody Production in Pristane-induced Lupus. The production of IgM anti-ssDNA antibodies 2–3 wk after treating BALB/c mice with pristane (2) appears to be thymus-independent (reference 2 and Richards, H.B., M. Satoh, J.C. Jennette, T. Okano, Y.S. Kanwar, and W.H. Reeves, manuscript submitted for publication). Because IL-6 can stimulate T cell-independent Ig production (22, 23), the induction of IgM anti-ssDNA antibodies by pristane in BALB/cAn IL-6^{-/-} and IL-6^{+/+} mice was investigated.

As shown in Fig. 1, IgM anti-ssDNA antibody levels were similar in IL-6^{-/-} versus IL-6^{+/+} mice 3 wk after pristane treatment. In contrast, IgG anti-ssDNA antibodies were detected at a high frequency 8 mo after pristane treatment only in IL-6^{+/+} mice ($P < 0.05$ versus IL-6^{-/-} by Mann Whitney test).

Anti-dsDNA antibodies are highly specific for lupus and are implicated in the pathogenesis of lupus nephritis. However, they were not detected previously in the 6-mo sera from pristane-treated BALB/c mice, a time when severe renal lesions already were apparent (2). In contrast, sera from 10 out of 26 BALB/cAn (IL-6^{+/+}) mice (38%) 8 mo after pristane treatment were strongly positive for anti-dsDNA antibodies by *Crithidia luciliae* kinetoplast staining (Table 1) at titers ranging from 1:80 to 1:640 (Table 2). Anti-dsDNA antibodies were not detected in pristane-treated IL-6^{-/-} mice or PBS-treated IL-6^{+/+} or IL-6^{-/-} mice (Table 1). Although the *Crithidia luciliae* kinetoplast staining assay is highly specific for anti-dsDNA activity, occasionally antihistone antibodies give a false positive reaction. To exclude that possibility, the sera were tested for

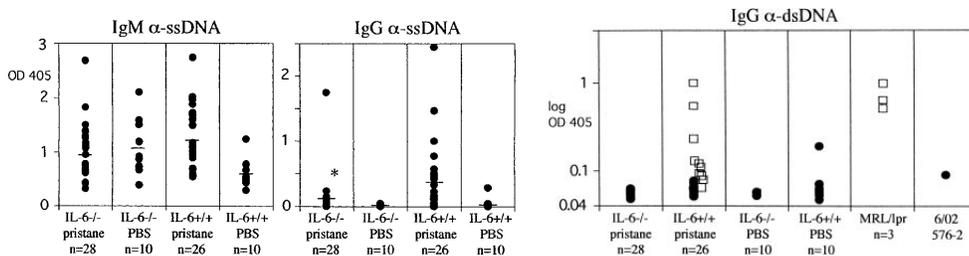


Figure 1. Anti-ssDNA and anti-dsDNA autoantibody levels. (left) Sera from BALB/cAn IL-6^{-/-} or IL-6^{+/+} mice 3 wk after pristane or PBS treatment were tested by ELISA for IgM anti-ssDNA antibodies at a dilution of 1:500. Sera from the same mice 8 mo after treatment were tested by ELISA for IgG anti-ssDNA antibodies. Symbols represent individual animals

and horizontal lines represent the mean $A_{405\text{ nm}}$ of each group (* $P < 0.005$ in the IL-6^{+/+} pristane versus IL-6^{-/-} pristane by Mann Whitney test). (right) Sera from BALB/cAn mice 8 mo after pristane or PBS treatment, 5-mo-old MRL/*lpr* mice, and mAb 6/O2 576-2 (IgG anti-ssDNA) were analyzed by ELISA for IgG anti-dsDNA antibodies at a 1:160 dilution. Samples were negative (●) or positive (□) positive by *Crithidia* assay at a 1:40 dilution.

Table 1. Frequency of Autoantibodies in Pristane-treated BALB/cAN Mice

Strain and Rx	No. of mice	Anti-nRNP/Sm*	Anti-Su*	Anti-dsDNA*
IL-6 ^{-/-} pristane	28	13 (46%)	10 (36%)	0
IL-6 ^{-/-} PBS	10	0	0	0
IL-6 ^{+/+} pristane	26	15 (58%)	8 (31%)	10 (38%)
IL-6 ^{+/+} PBS	10	0	0	0

*Sera from BALB/cAn mice were tested 8 mo after treatment with pristane or PBS. Anti-nRNP/Sm and Su antibodies were detected by immunoprecipitation and their presence was confirmed by ELISA (Fig. 3). Anti-dsDNA antibodies were detected in the sera by *Crithidia luciliae* kinetoplast staining at a dilution of 1:20.

IgG anti-dsDNA antibodies by ELISA. The ELISA was somewhat less sensitive than the fluorescence assay, since only sera with titers >1:640 gave a significantly stronger reaction by ELISA than the *Crithidia*-negative group. An anti-ssDNA mAb (6/O2 576-2; from Dr. D. Pisetsky, Duke University, Durham, NC) also showed weak ELISA reactivity. Remarkably, none of the sera from pristane-treated IL-6^{-/-} mice bound to S1 nuclease-treated DNA at 8 mo (Fig. 1).

Together, these results indicate that pristane-treated BALB/cAn mice develop high levels of IgG anti-ssDNA and anti-dsDNA antibodies, and that these autoantibodies are IL-6 dependent. However, the appearance of these autoantibodies is delayed until 8 mo after treatment, which is after the onset of renal disease. Some of the pristane-treated BALB/cAn mice developed IgG anti-dsDNA antibodies at levels comparable to those in MRL/*lpr* mice (Table 2, Fig. 1). Although it has been suggested that anti-dsDNA antibody production can result from polyclonal B cell activation (24), the marked polyclonal hypergammaglobulinemia seen in both IL-6^{+/+} and IL-6^{-/-} mice 3–5 mo after pristane treatment was not accompanied by an anti-dsDNA response, suggesting that other mechanisms may be more important in generating these autoantibodies. These may include effects of IL-6 on terminal B cell differentiation (17, 25).

Table 2. Titers of Anti-dsDNA Antibodies in Pristane-treated BALB/cAN IL-6^{+/+} Mice*

Titer	No. of mice
1:80	1
1:160	4
1:320	2
≥1:640	3

**Crithidia luciliae* kinetoplast staining assay.

IgG Antichromatin Antibodies. Like anti-dsDNA, anti-chromatin antibodies are found at high levels in MRL/*lpr* and other lupus mice (15, 26). Anti-DNA responses are linked to the immune response to chromatin, since T cells specific for histones can help B cells producing anti-DNA antibodies (27). Thus, it was of interest to see if antichromatin and anti-DNA antibodies exhibit similar IL-6 dependence. Pristane induced high levels of IgG antichromatin antibodies within 3–5 mo, but only in BALB/cAn IL-6^{+/+} mice (Figs. 2 and 3, C and D). Interestingly there was low level spontaneous production of these autoantibodies in aged IL-6^{+/+} PBS-treated mice (Fig. 2), which was absent in IL-6^{-/-} animals. Thus, IL-6 may be important for the production of pristane-induced, as well as spontaneous, IgG antichromatin autoantibodies.

Since IL-6 plays an important role in the differentiation of antigen plus CD40 coactivated B cells into antibody-secreting cells (28), the lack of IgG anti-DNA/chromatin autoantibodies in IL-6-deficient mice may reflect an absolute requirement for IL-6 in the differentiation of germinal center B cells into anti-DNA/chromatin secreting plasma cells. This interpretation is consistent with previous observations that IL-6 is crucial for the development of anti-DNA/chromatin antibodies in spontaneous murine lupus models (6, 7) and that the production of these autoantibodies also is dependent on CD40-CD40 ligand interactions (29, 30). Rheumatoid factor is not produced in CD40 ligand-deficient MRL/*lpr* mice either, whereas anti-nRNP/Sm antibodies are (30), suggesting that the latter may be regulated differently than anti-DNA and rheumatoid factor. Like anti-dsDNA, rheumatoid factor was induced by pristane in IL-6^{+/+} but not IL-6^{-/-} mice (data not shown), suggesting that similar regulatory mechanisms may exist in pristane-induced lupus.

IgG Anti-nRNP/Sm and Anti-Su Antibodies. The IL-6 dependence of IgG anti-DNA and chromatin autoantibodies is consistent with previous observations in the (NZB/

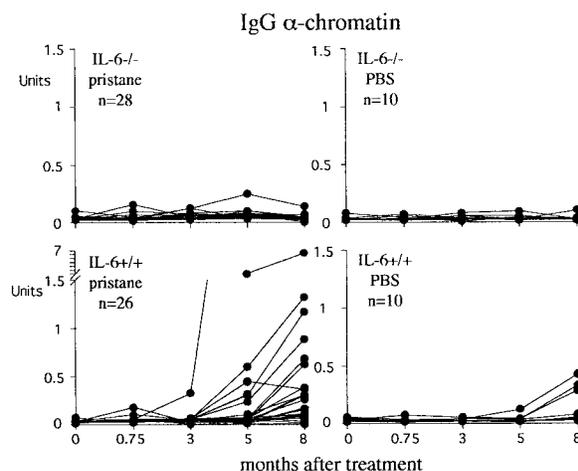


Figure 2. IgG antichromatin antibody ELISA. Serial serum samples from all pristane- or PBS-treated mice were analyzed at a dilution of 1:500 for IgG anti-chromatin antibodies by ELISA. Levels of IgG antichromatin are expressed in units × 10⁶ (see Materials and Methods).

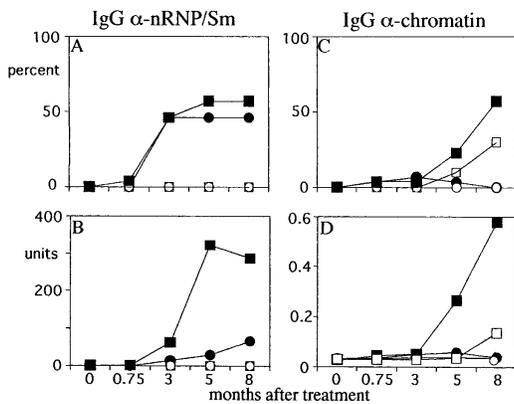


Figure 3. Anti-nRNP/Sm and anti-chromatin antibody ELISAs. Sera from pristane treated IL-6^{-/-} ($n = 28$, ●) or IL-6^{+/+} ($n = 26$, ■) mice and from PBS-treated IL-6^{-/-} ($n = 10$, ○) or IL-6^{+/+} ($n = 10$, □) mice 0–8 mo after treatment were tested for anti-nRNP/Sm (A and B) and anti-chromatin (C and D) antibodies by ELISA. (Top) Frequencies (%) of sera containing IgG anti-nRNP/Sm (A) or anti-chromatin (B) autoantibodies (defined as >3 SDs above the mean of negative controls). (Bottom) Levels of anti-nRNP/Sm (B) or anti-chromatin (D) autoantibodies (in units). Data represent the mean of all animals in each group.

W)F1 lupus model (6, 7). However, (NZB/W)F1 do not produce other autoantibodies characteristic of lupus, such as anti-nRNP/Sm. Thus, it is not known whether or not the latter also are IL-6 dependent. This question was addressed in the pristane-induced lupus model, in which autoantibodies to both RNA-protein and DNA-protein autoantigens are produced.

In contrast to the absence of IgG anti-dsDNA and anti-chromatin autoantibodies and in IL-6^{-/-} mice (Figs. 1 and 2, Table 1), the frequencies of anti-nRNP/Sm autoantibodies were comparable in the IL-6^{-/-} and IL-6^{+/+} mice (Fig. 3 A, Table 1), although levels were lower in the IL-6^{-/-} mice (Fig. 3 B). Similarly, the frequencies of anti-Su autoantibodies in IL-6^{+/+} versus IL-6^{-/-} mice were comparable (Table 1). In agreement with the ELISA data, both anti-nRNP/Sm and anti-Su autoantibodies were detectable in the sera of IL-6^{-/-} by immunoprecipitation of ³⁵S-labeled cell extracts. We conclude that the induction of anti-nRNP/Sm and Su autoantibodies is IL-6 independent, whereas their levels are influenced by IL-6. Since IgG2a was the predominant isotype of the anti-nRNP/Sm and Su, as well as anti-dsDNA and -chromatin autoantibodies (not shown), it is unlikely that the differential effects of IL-6 on their induction reflects merely an altered ability to produce antibodies of a particular isotype or isotypes. Furthermore, analysis of total Ig levels revealed marked increases in IgG2a, IgG2b, and IgG3 from 1 to 3 mo after pristane treatment both in IL-6^{+/+} and IL-6^{-/-} mice (data not shown). This was only slightly less dramatic in the IL-6^{-/-} group than in the IL-6^{+/+} mice.

Although DNA binding is critical for the activation of a subset of anti-Sm B cells (31), our data argue that the induction of anti-DNA and anti-Sm responses may be at least partially independent processes. This also appears true in CD40 ligand-deficient mice (30). Whereas anti-DNA/chromatin is

CD40 and IL-6 dependent, anti-nRNP/Sm antibody production may be enhanced via a T cell-dependent and CD40- and IL-6-independent mechanism (Richards, H.B., M. Satoh, J.C. Jennette, T. Okano, Y.S. Kanwar, and W.H. Reeves, et al., manuscript submitted for publication). Certain CD40 ligand-negative T cell clones retain the capacity to induce plasma cell differentiation (32). Recently, it was shown that the CD27-CD70 signaling pathway may promote B cell differentiation into IgG-secreting plasma cells, whereas CD40 ligand-CD40 signaling may be more important for maintaining B cell proliferation and differentiation into memory cells (33, 34). The role of this or other pathways of CD40 ligand-independent B cell activation in the generation of anti-nRNP/Sm and Su antibodies remains to be determined.

IL-6 is thought to influence the development of spontaneous human and murine lupus. It may play a role in the induction of autoantibodies (17) and nephritis (35, 36), as well as in polyclonal B cell activation (37, 38). Polyclonal hypergammaglobulinemia in lupus may reflect both the overexpression of IL-6 receptors on B cells (37, 39) and, in MRL/*lpr* mice, increasing IL-6 gene expression and serum levels with age (40). Although increased IL-6 production in MRL mice is related to the presence of the *lpr* gene, it is not produced by the abnormal double negative T cells (40), and may be derived from the expanded macrophage subset of these mice (6, 41).

Summary and Clinical Implications. The goal of this study was to examine the production of anti-dsDNA antibodies, long considered to be a marker of lupus nephritis (4, 5), in pristane-induced lupus. Anti-dsDNA antibodies were produced late in the course of pristane-induced lupus and were strictly IL-6 dependent, possibly reflecting the IL-6 overproduction characteristic of pristane-treated mice (8, 9). However, their onset at 8–11 mo after pristane treatment was well after the establishment of renal disease (2), arguing that the renal lesions may not be caused directly by anti-dsDNA antibodies. Nevertheless, renal disease in pristane-treated B6 IL-6^{-/-} mice is milder than in IL-6^{+/+} controls (Richards, H.B., M. Satoh, M. Shaw, C. Libert, V. Poli, and W.H. Reeves, unpublished data), consistent with the idea that IL-6 itself may contribute to the development of nephritis (42). Interestingly, although the levels of anti-nRNP/Sm antibodies also were influenced by IL-6, their frequency was not, suggesting that they may be regulated differently from anti-DNA antibodies.

The different cytokine requirements for anti-dsDNA and anti-Sm antibody production may help to explain differences in the patterns of their expression seen clinically. For example, low levels of anti-chromatin antibodies developed spontaneously in aged BALB/cAn^{+/+} mice but not in IL-6^{-/-} mice (Fig. 2), an observation that may be relevant to the increased frequency of antinuclear antibodies (43) and higher IL-6 production (44) in elderly people.

The extreme variability characteristic of anti-dsDNA antibody levels (5) contrasts with the fact that the levels of anti-Sm generally remain relatively stable over time. We speculate that, as in pristane-induced lupus, anti-dsDNA antibody production in human SLE may be IL-6 depen-

dent. Thus, anti-dsDNA/chromatin antibody levels might be a surrogate marker for increased IL-6 production, which is associated with active SLE (37). The induction of anti-DNA antibodies in mice by lipopolysaccharide (45), a potent inducer of IL-6 (46), raises the possibility that microbial stimulation could trigger both IL-6 production

and anti-DNA autoantibody production. Indeed, pristane-induced lupus is milder in specific pathogen-free than in conventionally housed mice (12). In view of the importance of anti-DNA antibodies and IL-6 in lupus nephritis, it may be of interest to examine the role of microbial stimulation of IL-6 production in the pathogenesis of lupus flares.

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