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D C Haynes, M E Gershwin

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Research Article

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Diversity of Autoantibodies in Avian Scleroderma

An Inherited Fibrotic Disease of White Leghorn Chickens

Darrell C. Haynes and M. Eric Gershwin
Division of Rheumatology-Clinical Immunology, Department of Internal Medicine, University of California at Davis, California 95616

Abstract. University of California, Davis line 200 White Leghorn chickens develop an inherited progressive fibrotic disease that includes the appearance of antinuclear antibodies (ANA). To further characterize these ANA, serial aged line 200 birds were studied. Greater than 50% of line 200 birds develop antinuclear and anticytoplasmic antibodies; fluorescent staining patterns included cytoplasmic spider web, most prevalent at 1 mo of age, and fine speckled patterns, characteristic of chickens 6 mo and older. By enzyme-linked immunosorbent assay, 40.4% of line 200 birds were found to have antibodies to single-stranded DNA (ssDNA). In contrast, antibodies to histones, RNA, or poly A · poly U were not detected. Precipitating antibodies to saline extracts from chicken liver were noted in 33.3% of line 200 birds. Saline extracts from turkey, pheasant, and partridge liver but not rat, rabbit, or mouse tissues were also positive in immunodiffusion testing with these line 200 birds. The antigenicity of chicken liver extracts was sensitive to pronase, protease K, and pH variations >10 and <5; however, they were resistant to trypsin, DNase, RNase, and incubation at 37°C and 56°C for 1 h. Cell fractionation in conjunction with column chromatographic techniques revealed that several protein antigens with apparent molecular weights in the range of 62,000–290,000 were present in cytoplasm but not in isolated nuclei. Line 200 sera were not reactive against nuclear ribonucleoprotein, Sm, Scl-70, or SS-B/La antigens. Thus, line 200 chickens develop antinuclear and anticytoplasmic antibodies at an early age, which recognize a unique group of protein antigenic determinants found only in avian species. Moreover, and of particular interest, the presence of autoantibodies to saline-extractable antigens correlated with positive ANA, antibodies in ssDNA, and to the clinical expression of disease.

Address reprint requests to Dr. Gershwin.
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Introduction

University of California, Davis (UCD) line 200 White Leghorn chickens develop a constellation of features including dermal and esophageal fibrosis, distal polyarthritis, microvascular alterations, and accelerated mortality (1). This syndrome appears within the first 6 wk after hatch, as swelling, erythema, and necrosis of the comb, digits, and skin. Birds that survive these initial insults develop mononuclear cell infiltration of esophagus, lung, testes, small intestine, and heart with prominent occlusion of small- and medium-sized blood vessels. Serologic abnormalities of line 200 birds include rheumatoid factors and antibodies to collagen and antinuclear antibodies (ANA).[1] In the present report we have serially monitored line 200 chickens to provide insight into the diversity of antinuclear antibodies. Moreover, on the basis of results from these observations, line 200 sera were studied to monitor reactivity to polynucleotides and saline-extractable antigens; physicochemical characterization was then used to define the nature of these antigenic systems.

We report herein that a significant proportion (65% at 6 mo of age) of line 200 chickens develop ANA, which react primarily in a fine speckled staining pattern. Anticytoplasmic antibodies (ACA), reacting in a cytoplasmic spider web staining pattern, were present in >50% of line 200 birds at 1 mo of age. Fur-

1. *Abbreviations used in this paper:* ANA, anti-nuclear antibodies; ACA, anti-cytoplasmic antibodies; CLE, chicken liver extract; dsDNA, double stranded DNA; ELISA, enzyme-linked immunosorbent assay; ENA, extractable nuclear antigens; HB, homogenizing buffer; IIF, indirect immunofluorescence; PAGE, polyacrylamide gel electrophoresis; PBS, 0.01 M phosphate buffer, 0.15 M sodium chloride, pH 7.4; PMSF, phenylmethylsulfonyl fluoride; RNP, nuclear ribonucleoprotein; SLE, systemic lupus erythematosus; ssDNA, single stranded DNA.

thermore, one third of line 200 birds expressed precipitating antibodies to protein antigens isolated from saline extracts of liver from avian species but not to rat, rabbit, or mouse tissues. Line 200 ANA show striking similarities to those found in human patients with connective tissue diseases (2–6). However, differences are present in antigenic specificities recognized in preparations of extractable nuclear antigens (ENA), which suggests that line 200 chickens might express some markers of autoimmunity in a form unique to avian species.

Methods

Birds. Inbred lines of White Leghorn chickens were provided by the Department of Avian Sciences, UCD. The natural history, as well as care and maintenance of UCD line 200 and control line chickens has been reported (1). Line 200 was outcrossed to line 011 to produce an F_1 generation, which was designated line 211. F_2 and F_3 generations of line 211 were designated line 206. Control chickens in this study included several highly inbred lines (003, 011, and 446) and one outbred line, 010. Characteristics, including genetic backgrounds and immunohematology, of these control lines have been extensively described (7).

Sera and plamsa. Samples of serum and plasma were collected by venipuncture from the jugular or wing vein at 1, 3, and 6 mo, and 1 yr of age from line 200, line 211, line 206, and control line chickens (1). Samples were heat inactivated at 56°C for 30 min and stored at −70°C. Thawed samples were cleared of lipid precipitates, which were found to form upon freeze-thawing, by centrifugation at 7,000 *g* for 2 min before use. Immunoglobulin purification was performed on chicken sera by a modification of the method of Benedict (8).

Reference sera to nuclear DNA, nuclear ribonucleoprotein (RNP), Sm, SS-B/La, and RNP plus Sm were obtained from the Centers for Disease Control, Atlanta, Georgia. In addition, reference sera to Scl-70 and anti-centromere antibody were donated by Dr. Eng Tan, Scripps Clinic and Research Foundation, La Jolla, CA.

Immunofluorescence. Indirect immunofluorescent studies were performed by using the human epithelial cell line HEp-2 as substrate (Antibodies, Inc., Davis, CA). Conjugates included goat anti-chicken gamma globulin (Cappel Laboratories, Cochranville, PA), goat anti-chicken μ-chain specific, (Pel-Freez Biologicals, Rodgers, AR), rabbit anti-chicken 7S heavy chain specific (donated by Dr. A. Benedict, University of Hawaii, Honolulu), and goat anti-mouse IgG or IgM specific (Meloy Laboratories, Inc., Springfield, VA). The goat anti-mouse IgG/IgM specific conjugate was used in conjunction with a monoclonal mouse anti-chicken IgA (donated by Dr. Max Cooper, University of Alabama, Birmingham, AL) in a triple-antibody sandwich technique. Chicken sera was applied first to the HEp-2 substrate followed by the monoclonal mouse anti-chicken IgA and then the goat anti-mouse IgG/IgM antiserum. Between each step there was a 10-min wash with 0.01 M phosphate buffer, 0.15 M sodium chloride, pH 7.4 (PBS). For all conjugates a 1+ staining (intensity graded from 1+ to 4+) at 1/20 dilution of sera was regarded as positive. This decision was on the basis of an unbiased analysis of 36 control sera with these same conditions.

Inhibition of immunofluorescence. Inhibition of nuclear staining was attempted by first incubating the HEp-2 substrate with human positive control sera having either anti-ENA, anti-Scl-70 or anti-single stranded DNA (ssDNA) binding activity before overlaying with chicken sera. Reductions in titer or changes in staining pattern were quantitated after incubation of the HEp-2 substrate at 4°C for 18 h with human ANA-positive sera. Absorption of line 200 sera was performed by incubating 75 μl of sera with either 75 μl of chicken liver extract (preparation described below) or 10 mg of mouse liver or rabbit thymus powder (Pel-Freez Biologicals). After a 24-h incubation at 4°C, sera were centrifuged at 7,000 *g* for 2 min and then tested at a 1/20 dilution for a change in immunofluorescent staining pattern.

Anti-centromere antibody. Antibodies directed to the centromeres of chromosomes were assayed by using chromosomal spreads prepared from chicken feather pulp (9). Indirect immunofluorescence with goat anti-chicken gamma globulin was carried out on 25 line 200 and 10 control line sera along with a positive human serum control for anti-centromere antibody.

Enzyme-linked immunosorbent assay (ELISA). ELISA was performed to detect antibodies to calf thymus histones, calf thymus RNA, poly A · poly U, single stranded calf thymus DNA (Sigma Chemical Co., St. Louis, MO). ssDNA was prepared by heating a solution of DNA at 1 mg/ml to 100°C for 10 min and then immediately immersing in an ice-water bath. Test antigens at 100 μg/ml were coated on 96-well polysytrene microtiter plates (Falcon Plastics, Oxnard, CA) in 0.1 M carbonate buffer, pH 9.6. After incubation at 4°C for 18 h, the test antigen solution was removed and plates were washed twice with PBS containing 0.05% Tween 20 (azide free). Plates were blocked with 1.0% bovine serum albumin (BSA) in the above washing buffer for 2 h at 22°C. After this blocking step, antigen coated-plates were once again washed and either used immediately or stored at 4°C for up to 1 mo.

Test sera were diluted 1:100 with a solution of 1.0% BSA in 0.05% Tween 20, PBS; and 100-μl aliquots were added to individual wells. Plates containing diluted sera were incubated overnight at 4°C, washed three times, and incubated with rabbit anti-chicken gamma globulin (E · Y Laboratories, San Mateo, CA) at a 1:500 dilution in 1.0% BSA, 0.05% Tween 20, and PBS. After additional washing, 5-aminosalicylic acid (Sigma Chemical Co.) was added for color development. The reaction was terminated after 1 h with 0.1 ml of 1 N NaOH and absorbances were read immediately with a MicroELISA reader (Dynatech Laboratories, Inc., Dynatech Corp., Alexandria, VA) at 450 nm or at 450/630 nm to lessen differences due to optical irregulatories in plate bottoms. Assays were performed in duplicate; controls included positive and negative sera, conjugate control, and substrate control.

Inhibition of ssDNA ELISA. 10 Line 200 serum samples, selected on the basis of high reactivity to ssDNA by ELISA, were incubated 18 h at 4°C with 50 μg of either ssDNA, double stranded DNA (dsDNA), RNA, cardiolipin, phosphatidyl ethanoloamine, poly A, poly C, poly G, or poly U (Sigma Chemical Co.). Cardiolipin and phosphatidyl ethanolamine were used as micelles in inhibition assays as described (10). Aliquots were diluted in 1.0% BSA in PBS containing 0.05% Tween 20, to obtain a 1:100 serum dilution; they were assayed as above. In addition, six high binding sera were incubated at 4°C for 18 h with varying amounts (0.01 μg–1.0 mg) of either ssDNA, dsDNA, RNA, or poly G before assaying for anti-ssDNA binding activity.

Immunodiffusion. A modification of the Ouchterlony double immunodiffusion method was used to demonstrate and identify precipitating antibodies in line 200 and control line sera. 0.6% agarose (Seakem Agarose, Marine Colloids, Inc., Springfield, NJ) in PBS containing 0.02% NaN_3 was pipetted into immunodiffusion plates (Miles Laboratories, Inc., Elkhart, IN) and wells of 5-mm diam were cut 3 mm apart. Plates were incubated at 22°C and precipitin reactions read at 24, 48, and 72 h. Appropriate controls for each tested antigen were included on each plate.

Ouchterlony analysis was used to characterize the antibody-antigen reactions of line 200 sera on the basis of similar studies in humans, to resolve complex patterns of reactivity to cellular antigens (5, 11). Immunoelectrophoresis that was performed according to the method of Mimori et al. (5) was not found to possess sufficient sensitivity. Similarly, due to the poor precipitability of line 200 antigenic specificities, a characteristic of chicken immunoglobulins, it was not possible to use immunoprecipitation. Finally, it should be noted that the combination of sodium dodecyl sulfate (SDS), 2-mercaptoethanol, and heat denatures the chicken liver extract. Thus, Western blots (12, 13) were not reproducible due to the loss of antigenicity.

Tissue extracts. Tissue extracts used in double immunodiffusion were prepared from rabbit thymus acetone powder, mouse liver powder, fresh frozen rat liver (Pel-Freez Biologicals) and fresh control chicken, turkey, pheasant, and partridge liver as well as chicken thymus. ENA from rabbit thymus acetone powder and mouse liver powder were prepared by extracting 90 mg/ml of powder with PBS at 4°C for 4 h with stirring (14). Extracts were centrifuged at 10,000 *g* for 30 min and stored at −20°C. Whole tissue extracts were prepared according to the method of Mimori et al. (5), with some modification. Rat, chicken, turkey, pheasant, and partridge liver and chicken thymus extracts were prepared by homogenization in a motor-driven Potter-Elvehjem homogenizer in an equal amount by weight of PBS with 1 mM phenylmethylsulfonyl fluoride (PMSF) at 4°C. The homogenate was stirred for 4 h at 4°C and then centrifuged at 10,000 *g* for 30 min with an additional centrifugation at 104,000 *g* for 90 min. The supernatant was aliquoted and stored at −20°C until needed. Saline extracts from chicken liver were designated CLE and used in further fractionation procedures.

Cellular fractionation of chicken liver into fractions designated nuclei-enriched, mitochondria-enriched, microsome-enriched, and cytoplasm was accomplished by differential centrifugation in isotonic sucrose (15). All procedures were carried out at 0–4°C. In brief, this method consisted of first washing, then mincing 100 *g* of liver tissue in eight vol of homogenizing buffer (HB) containing 0.25 M sucrose, 5 mM magnesium acetate, and 1 mM PMSF. In aliquots of 25–30 ml the minced liver tissue was homogenized using a motor driven Potter-Elvehjem homogenizer (four strokes). All aliquots were pooled; and nuclei were pelleted at 700 *g*; the supernatant was centrifuged twice more at 700 *g* to pellet residual nuclei and then centrifuged at 5,000 *g* for 10 min to pellet mitochondria. The resultant supernatant from this fraction was centrifuged at 57,000 *g* for 60 min to pellet microsomes. The final supernatant was designated cytoplasm. Nuclei, mitochondria, and microsome fractions were washed a total of four times in HB then resuspended in PBS. Suspensions of nuclei and mitochondria were sonicated on ice in six 15-s pulses using a sonicator (Heat Systems-Ultrasonics, Inc., Plainview, NY) at a setting of six. The sonicates were stirred at 4°C for 1 h then centrifuged at 104,000 *g* for 30 min. Supernatants were stored at −20°C.

Enzymatic, heat, and pH treatments. Enzymatic digestion of CLE was performed at an enzyme-to-substrate ratio of 1:10 (11) after first determining the protein concentrations of the samples to be treated (16). Extracts were adjusted to 2 mg/ml of protein in PBS and either trypsin, pronase, protease K, ribonuclease (RNase), or deoxyribonuclease (DNase) I (Sigma Chemical Co.) was added to give an enzyme concentration of 0.2 mg/ml. All digestions took place at 37°C for 1 h. Reactions were terminated at 0–4°C and 1 mM PMSF was added to the trypsin, pronase, and protease K-digested extracts. Immunodiffusion was used to test for residual antigenicity at 22° and 4°C; untreated control samples were included on each plate.

The sensitivity of CLE to heating was determined by incubation at 56°C for 1 h and 37°C for 1 h. Extracts were cleared of precipitates by centrifugation at 2,000 *g* for 10 min, and then tested by immunodiffusion. The effect of pH variations on the antigenic activity of CLE was investigated by dialysis of 0.5-ml aliquots against buffers of various pH at 4°C for 48 h; glycine/HCl (pH 2.0–3.0), acetate (pH 4.0–5.0), phosphate (pH 6.0–8.0) and glycine/NaOH (pH 9.0–11.0). All aliquots were neutralized by dialysis against PBS, pH 7.4, for 24 h. Residual activity was tested by immunodiffusion.

Gel filtration. Crude CLE or DEAE-Sephacel-chromatographed CLE was fractionated through a calibrated Sephacryl S-300 superfine (Pharmacia Fine Chemicals AB, Uppsala, Sweden) column 2 × 100 cm. The running buffer used to fractionate CLE consisted of 0.01 M Tris-HCl, which contained 0.4 M NaCl with 0.005 M 2-mercaptoethanol, pH 7.4. Fractions of 2 ml each were collected and tested for antigenic activity by immunodiffusion.

Ion-exchange chromatography. Ion-exchange chromatography of the CLE was performed with DEAE-Sephacel (Pharmacia Fine Chemicals) equilibrated with an initial buffer of 0.01 M Tris, 0.0 M NaCl, pH 8.4. The CLE was dialyzed against the initial buffer and then eluted by using a linear NaCl gradient having a final buffer concentration of 0.3 M NaCl in 0.01 M Tris, pH 8.4. This was then followed by step gradients of 0.5 and 1.0 M NaCl. Antigenic activities of each fraction were tested by double immunodiffusion. Positive fractions were pooled and concentrated by ultrafiltration (Amicon Corp., Lexington, MA).

SDS-polyacrylamide gel electrophoresis (PAGE). SDS-PAGE was performed according to Laemmli (13) with 10 or 12% acrylamide slab gels. Samples were diluted in sample buffer consisting of 125 mM Tris-HCl, pH 6.8, with 8% SDS, 5% 2-mercaptoethanol, 20% glycerol, and bromophenol blue added as a tracking dye; samples were then boiled for 3 min before electrophoresis. Stacking was carried out at 40 V for 2 h, and then samples were electrophoresed at 25 mA for 4 h. Gels were stained with 0.02% Coomassie Brilliant Blue followed by exhaustive destaining in 10% methanol and 7% acetic acid solution overnight.

Correlation studies. Results from immunodiffusion testing of line 200 sera against CLE were correlated with the presence of ANA, which was demonstrated by indirect immunofluorescence and with antibodies to ssDNA determined by ELISA along with total comb involution, an outward manifestation of early disease expression. 24 sera samples, 12 positive for precipitating antibodies to CLE and 12 negative for such antibodies, were used in this study.

Results

Immunofluorescence studies. The frequencies of ANA and ACA in line 200 chickens are shown in Fig. 1. At 1 mo of age the predominant indirect immunofluorescence (IIF) staining pattern in line 200 was cytoplasmic spider web (Fig. 2 *A*). In addition

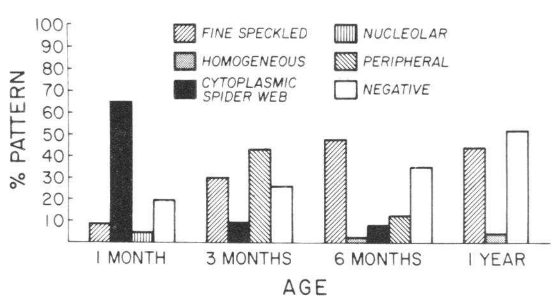

Figure 1. Incidence of staining patterns determined for line 200 sera at 1, 3, and 6 mo for 1 yr of age by indirect immunofluorescence of HEp-2 substrate.

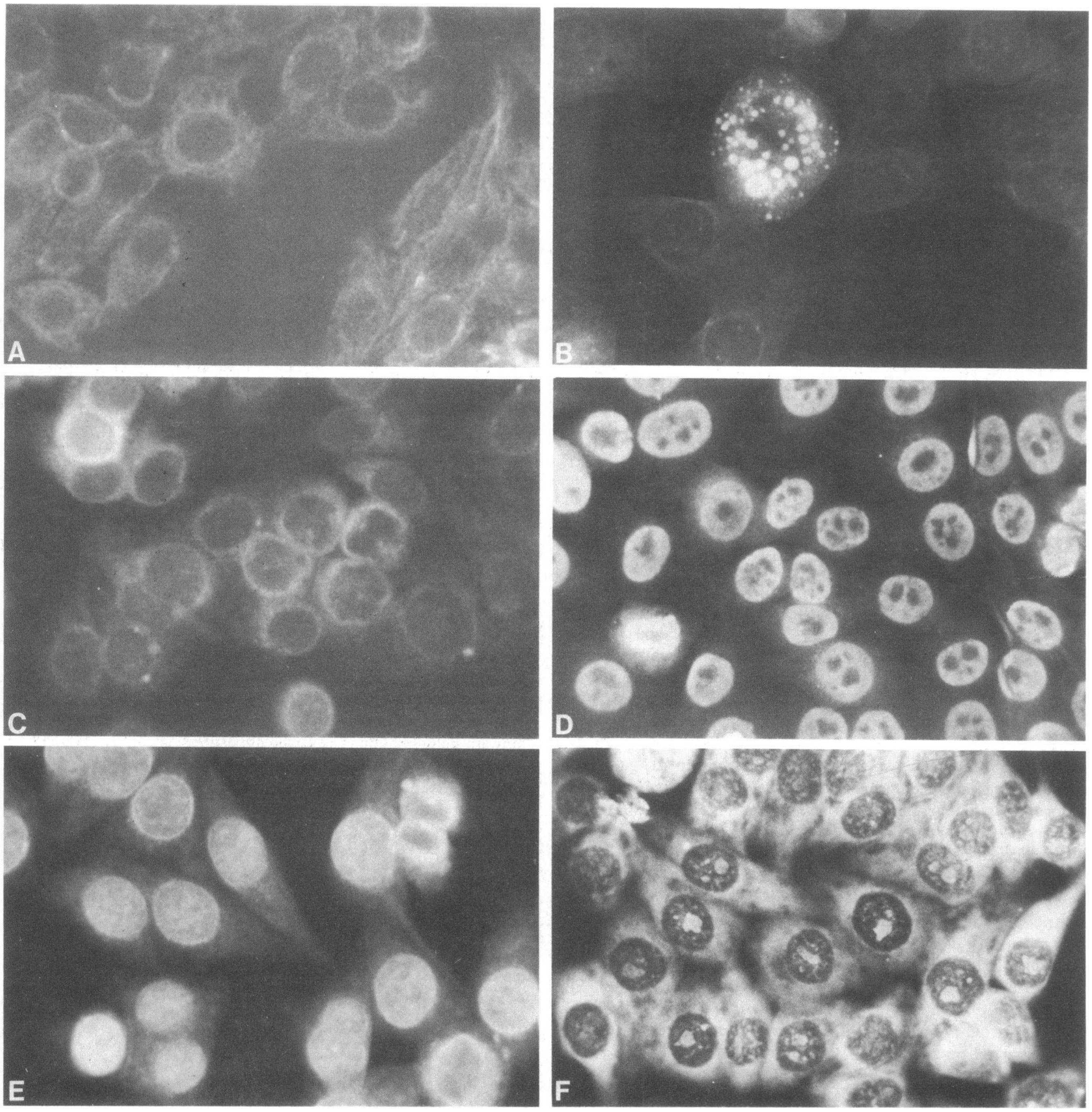

Figure 2. Representative nuclear and cytoplasmic staining patterns produced by line 200 sera using IIF with HEp-2 cells. *A* shows cytoplasmic spider web pattern; large discrete speckles present in mitotic figures are shown in *B*. *C* shows peripheral staining that appears almost cytoplasmic in origin. *D* shows diffuse fine speckles throughout the nucleus. *E* shows homogenous nuclear staining that is granular in appearance. *F* is a combination of nucleolar staining with discrete coarse nuclear speckles and cytoplasmic staining (× 310).

to being characterized by fine cytoplasmic staining, mitotic figures were noted to have very large discrete granules or speckles of varying diameter throughout the cytoplasm (Fig. 2 *B*). The incidence of this pattern decreased from 65% to <10% by 6 mo of age. At 3 mo of age the predominant staining pattern observed was peripheral (42%) (Fig. 2 *C*), which was closely followed in frequency by a pattern of granular fine speckles (30%). In birds 6 mo of age and older, diffuse fine speckled staining patterns were noted most often (Fig. 2 *D*). However, in many instances, the staining patterns were more complex than those shown in Fig. 2 *A–E* because of multiple patterns; these often included cytoplasmic spider web with diffuse fine speckles or as in Fig. 2 *F*, cytoplasmic staining in combination with nucleolar and large discrete speckles. Several sera produced fine speckled staining with packing so dense that this pattern could be mistaken for homogeneous nuclear staining. Moreover, the type of peripheral staining observed in line 200 birds 3–6 mo of age was not of the type seen in systemic lupus erythematosus (SLE), which appears more as a thin peripheral band of staining that lies within the nucleus on the HEp-2 substrate. In comparison, the peripheral staining pattern in line 200 appears more diffuse, with cytoplasmic staining that surrounds the nucleus giving a perinuclear halo with an almost cottonlike appearance. In contrast to the frequent appearance of positive IIF in line 200 chickens (52% at 12 mo, 65% at 6 mo) only 1 of 36 control line birds (line 003) was found to have a positive ANA, titer = 1/20.

Titers of anti-cellular antibodies (both nuclear and cytoplasmic) using anti-chicken gamma globulin conjugate, were highest at 6 mo of age with a mean ($\log_2$) of 6.48±1.67 and a range of 1:20–1:640. The mean ($\log_2$) titer at 12 mo of age was 6.07±1.54, while line 200 birds at 1 mo of age had a mean ($\log_2$) titer of 5.18±1.86.

Isotype distribution of line 200 ANA and ACA. Line 200 ANA and ACA were distributed across all chicken isotypes; this included 7S Ig, IgM, and IgA for all age groups (Table I).

IIF inhibition. Preincubation of the HEp-2 substrate with human sera positive for ENA, ssDNA, or Scl-70 failed to inhibit line 200 ANA. Absorption of ANA-positive line 200 sera giving homogeneous or fine speckled patterns with rabbit thymus powder was found to abolish immunofluorescent staining. Neither CLE nor mouse liver powder were found to give similar results. However, the nucleolar staining of ANA having a homogeneous pattern was inhibited by both CLE and mouse liver powder. Thus, the homogeneous ANA pattern of line 200 sera is due to at least two specificities with only the nucleolar specific antigen being present in saline extracts of chicken liver.

Table I. Isotype Distribution of Line 200 ANA and ACA

Age	Fluorescein isothiocyanate conjugate			
	Anti-gamma globulin	Anti-7s Ig	Anti-IgM	Anti-IgA
	%	%	%	%
1 mo	64.3	53.6	50.0	50.0
6 mo	65.0	82.0	77.5	60.0
12 mo	52.0	64.0	76.0	50.0

The values represent the percentage of line 200 chicken positive in the indirect immunofluorescent assay on HEp-2 cell substrate by age group and isotype specificity.

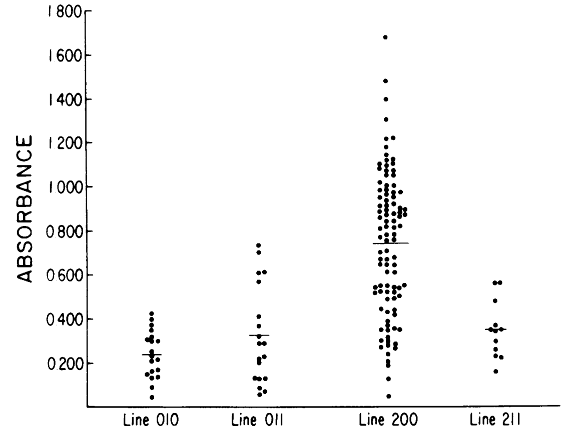

Figure 3. Antibodies to ssDNA in lines 200 and 211 and control lines 010 and 011 determined by ELISA. Each point represents the mean value of duplicate samples; absorbance was read at 450 nm.

Anti-centromere antibodies. Anti-centromere antibody was not found in 25 line 200 chickens ranging in age from 1 mo to 1 yr of age screened with the chicken feather pulp chromosomal spread preparation.

Anti-ssDNA ELISA. The majority of line 200 serum samples was found to have elevated levels of binding to ssDNA when compared with control sera; 53% were greater than 2 SD from the line 011 mean and 74% were greater than 2 SD from the line 010 mean (Fig. 3). The inclusion of line 211 and line 206 in this assay was with the intent of investigating the mode of inheritance of the line 200 anti-ssDNA response. As shown in Fig. 3, line 211, an F_1 generation of line 200, has a mean response not significantly different from controls. This initial screening for antibodies to ssDNA used birds 6–12 mo of age. In a subsequent time trial, line 200, line 206, and line 010 were followed from 1 to 6 mo of age (Table II). The mean response for line 200 sera in the ssDNA ELISA reached a plateau by 3 mo of age; at 6 mo responses had not altered significantly. Comparisons between lines at the 6 mo time point for pooled data in Table II show significant differences between means. Line 200 and 206 means were significantly elevated from the line 010 mean as calculated by the *t* test with *P* values < 0.001 and <0.05, respectively. Moreover, a significant difference was also noted in the percentage of chickens for each line with ssDNA binding values > 2 SD from the line 010 mean. Line 200 had 40.4%, while line 206 had 15.4% of tested individuals with test values > 2 SD from the control mean. There were no line 010 birds with test values > 2 SD from the control mean. Line 206, composed of F_2 and F_3 generation line 200 birds, had anti-ssDNA responses intermediate between controls and line 200.

ELISA to histones, RNA, and poly A · poly U. Antibodies directed to calf thymus total histones, calf thymus RNA, or poly A · poly U were not detected by ELISA in 19 line 200 sera

Table II. Detection of Antibodies to ssDNA in Chickens 1, 3, and 6 Mo of Age using Solid Phase ssDNA in an ELISA

	ssDNA binding values		
	1 mo	3 mo	6 mo
Line 200	0.178±0.020 (34)	0.503±0.032 (46)	0.504±0.027 (42)
Line 206	0.445±0.033 (16)	0.362±0.038 (27)	0.408±0.049 (13)
Line 010	—	—	0.238±0.027 (17)

The values are the means±SEM for absorbance readings at 450 nm/630 nm in the anti-ssDNA ELISA. In parentheses are the number of birds in each group. Statistical analysis of the line 200 and line 206 means at the 6-mo time point by the *t* test indicated significant differences in comparison with control line 010; $P < 0.001$ for line 200 and $P < 0.05$ for line 206.

and 5 control line 011 sera. Mean absorbancy values±SEM were 0.087±0.022 and 0.035±0.009 for histones, 0.059±0.016 and 0.026±0.007 for RNA, and 0.089±0.020 and 0.041±0.009 for poly A · poly U in line 200 and line 011, respectively. The values for line 200 sera were slightly, but not significantly, greater than control line responses in these three assays.

ssDNA ELISA inhibition studies. Inhibition studies of sera positive to ssDNA by ELISA performed with cardiolipin, phosphatidyl ethanolamine (analogues of the DNA backbone), poly A, poly G, poly U, poly C, RNA, dsDNA, and ssDNA demonstrated that the most efficient inhibition was produced by ssDNA. Inhibition was near 100% with ssDNA at 50 μg/ml; other nuclear antigens that produced significant inhibition included poly G (36%) and RNA (28%). Less than 10% inhibition was noted for cardiolipin, phosphatidyl ethanolamine, poly A, poly U, and poly C. Inhibition was also observed by changing the amount of inhibitor from 10^{-2} μg/ml up to 10^{3} μg/ml as shown for line 200 sera in Fig. 4. Inhibition was maximal for these line 200 sera samples in the anti-ssDNA ELISA with much lower levels of ssDNA than dsDNA, RNA, or poly G. For example, with bird number 10966 (Fig. 4 *A*) 50% inhibition was produced with 0.3 μg/ml of ssDNA, while 30.0 μg/ml of RNA, 6.0 μg/ml of poly G, and 200 μg/ml of dsDNA were required for equivalent inhibition.

Immunodiffusion analysis of CLE. Saline extracts from chicken thymus and chicken liver produced precipitin reactions with line 200 sera (Fig. 5 *A*). Similar reactions with line 200 sera were also noted for saline extracts isolated from the liver of turkey, pheasant, or partridge (Fig. 5 *B*). 18 out of 54 (33.3%) of line 200 chickens at 6 mo of age were positive for anti-CLE antibodies. In contrast, only 21.4% (3/14) line 206 were positive and 0/28 line 010 controls were positive for anti-CLE antibodies. Line 200 sera did not possess precipitating antibodies towards saline-extractable antigens found in rat or mouse liver or rabbit thymus (Fig. 5*A*). Anti-CLE activity of line 200 sera was abrogated by prior absorption with CLE; absorption with mouse liver powder or rabbit thymus powder did not have any effect on anti-CLE activity. Ouchterlony analysis of line 200 sera with normal chicken serum proteins including purified chicken immunoglobulins demonstrated that the anti-CLE activity was not due to reactivity against chicken serum proteins. Patterns of immunological reactivity in double-diffusion analysis suggest that at least four antigens present in CLE are recognized by line 200 sera. This was based on Ouchterlony analysis of crude CLE with selected line 200 immunoglobulin fractions, which produced reactions of identity, partial identity, nonidentity, plus multiple precipitin lines of reactivity. Furthermore, fractionation of chicken liver homogenates into nuclei, mitochondria, microsomes, and cytoplasm demonstrated that the antigenic activities lie within the cytoplasm and not the other cellular compartments (Fig. 6).

The autoantibodies in line 200 chickens were shown to be unique in comparison to those found in patients with connective tissue diseases. Sera specific for RNP, Sm, SS-B/La, and Scl-70 antigenic reactions were positive when tested against rabbit thymus extracts but only to RNP and Sm antigens in whole CLE.

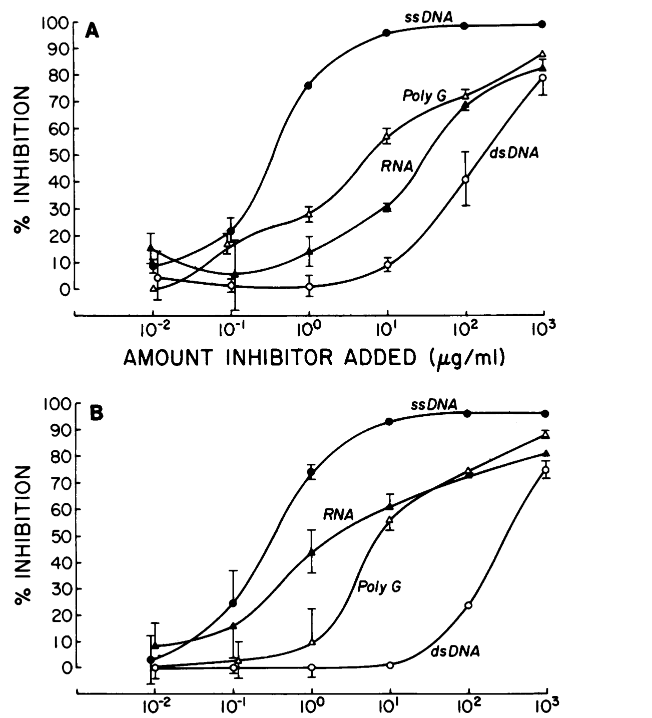

Figure 4. Inhibition of anti-ssDNA ELISA by ssDNA, dsDNA, RNA and poly G. Concentrations of polynucleotides varied from 10^{-2} μg/ml to 10^{3} μg/ml. Each value represents the mean ± SEM of triplicate samples. (*A*) Line 200, bird 10966; (*B*) line 200, bird 12820.

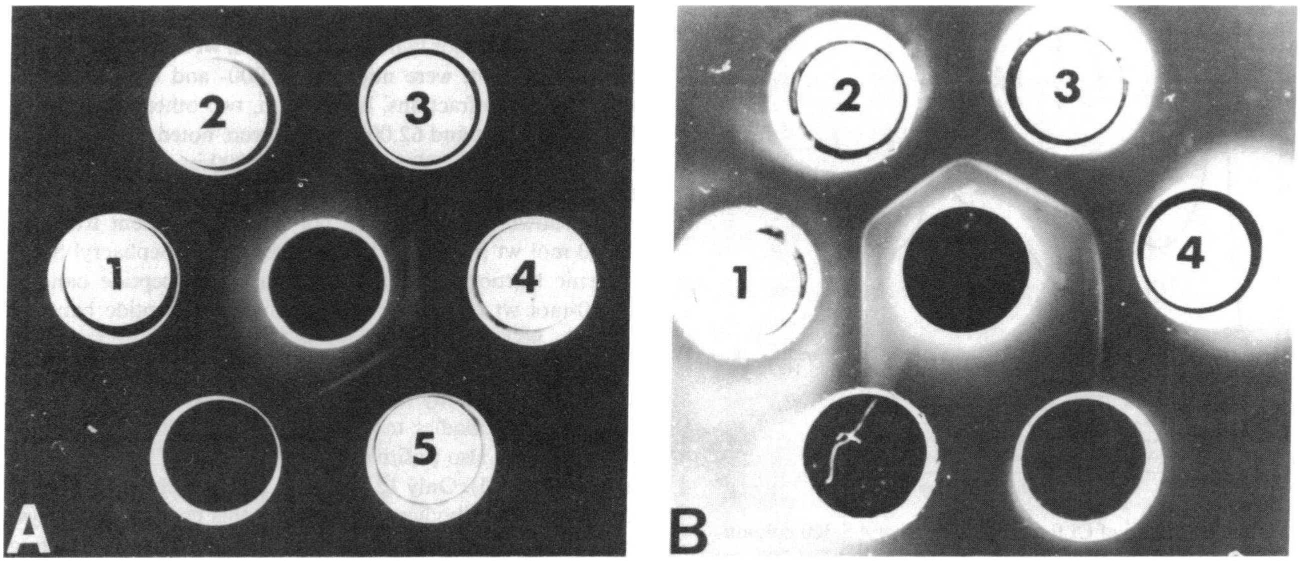

Figure 5. (*A*) Immunodiffusion of line 200 immunoglobulin fraction in center well against saline extracts from rabbit thymus (*1*), rat liver (*2*), mouse liver (*3*), chicken liver (*4*), and chicken thymus (*5*). Precipitin lines appear only against saline extracts from chicken liver and chicken thymus. (*B*) Immunodiffusion of line 200 immunoglobulin fraction in center well against saline extracts from chicken liver (*1*), turkey liver (*2*), pheasant liver (*3*), and partridge liver (*4*). Note lines of identity against saline liver extracts from chicken, turkey, pheasant and partridge.

CLE fractionated by gel filtration and reactive with line 200 sera did not produce precipitin reactions with positive patient control sera for anti-RNP and anti-Sm; although these antigens were present in crude CLE, they were not present in the partially purified CLE. Line 200 chickens recognize a unique group of antigenic determinants found so far only in avian species, moreover, they appear to be different from those identified in human patients with connective tissue diseases.

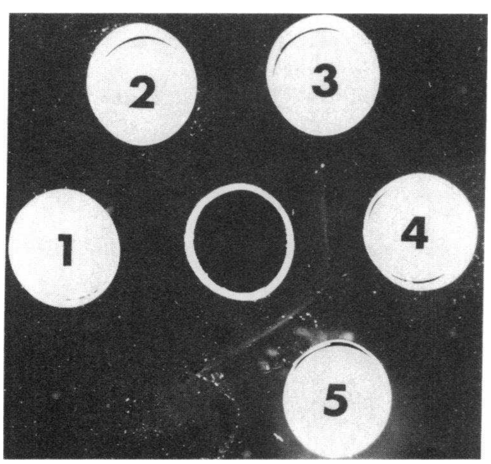

Figure 6. Immunodiffusion of line 200 immunoglobulins in center well against CLE fractionated into nuclei (*1*), mitochondria (*2*), microsomes (*3*), cytoplasm (*4*) and crude CLE (*5*).

Sensitivity of chicken liver extracts. Aliquots of crude CLE treated by enzymatic, heat, pH or chemical means revealed that for all sera samples tested, the antigenic determinants involved were insensitive to trypsin, DNase, RNase, 56°C for 1 h, 37°C for 1 h, and pH variations between 6.0 and 8.0. Antigenic reactivity of these antigens was abolished by treatment with pronase, protease K, and pH variations >10 and <5. Although all antigens were stable between pH 6.0 and 8.0, one was found to be stable from pH 5.0 to 10.0 for two individual line 200 serum samples.

Characterization of CLE antigens by gel filtration and ion-exchange chromatography. Gel filtration of crude CLE on a calibrated 2 × 100-cm Sephacryl S-300 superfine column revealed multiple peaks of antigenic activity (Fig. 7). This finding was based on the observation of differential reactivity by individual serum samples on the Sephacryl S-300 fractions. Estimated molecular weights for five of these peaks were ~290,000, 250,000, 160,000, 105,000, and 62,000. The relative incidence of each molecular weight specie in CLE-positive line 200 sera ranged from a high of 91.3% for the 250,000-mol wt peak to 21.7% for the 160,000- and 62,000-mol wt peaks. 78.3% of CLE positive line 200 sera were reactive with the 290,000-mol wt peak while only 30.4% reacted with the 105,000-mol wt peak.

DEAE-Sephacel chromatography of crude CLE yielded two peaks of activity when tested by immunodiffusion, fractions 34–48 and fractions 80–108 (Fig. 8). Antigenic activity was eluted at NaCl concentrations of ~0.1 and 0.3 M. Fractions corresponding to each antigenic peak were concentrated, and then subjected to gel filtration on Sephacryl S-300 as above. Gel filtration of the 0.1 M NaCl-eluted DEAE-Sephacel fractions

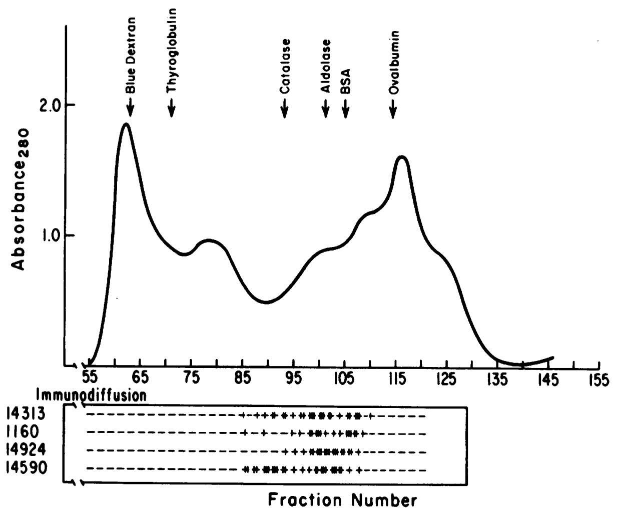

Figure 7. Gel filtration of CLE through a Sephacryl S-300 column (2.0 cm × 100 cm). Immunodiffusion of four selected line 200 sera demonstrated multiple peaks of antigenic activity with estimated molecular weights in the range of 62,000–290,000.

followed by immunodiffusion testing demonstrated two peaks of activity of 80,000- and 58,000-mol wt (Fig. 9 *A*). Similarly, when the 0.3 M NaCl-eluted DEAE-Sephacel fractions were sized by gel filtration, a single antigenic peak of 250,000-mol wt was noted (Fig. 9 *B*).

SDS-PAGE of CLE. SDS-PAGE analysis under reducing and denaturing conditions was performed on the 58,000-, 80,000-, and 250,000-mol wt CLE antigens purified by DEAE-Sephacel and Sephacryl S-300 chromatography (Fig. 10). Two polypeptides, a major band at 48,000-mol wt and a minor band at 45,000-mol wt, were noted in 58,000- and 80,000-mol wt Sephacryl S-300 fractions. In addition, two other polypeptides of 33,000-mol wt and 62,000-mol wt were noted in the 80,000-mol wt Sephacryl S-300 fraction; the 33,000-mol wt polypeptide represented the major protein in this antigen fraction (Fig. 10 *A*). As shown in Fig. 10 this polypeptide is absent from the 58,000-mol wt fraction. The 250,000-mol wt Sephacryl S-300 antigenic fraction contained one major polypeptide band of 63,000-mol wt plus at least four minor polypeptide bands at 136,000-, 96,000- 45,000-, and 38,000-mol wt.

Correlation between serologic assays in line 200 chickens. A retrospective study of 24 line 200 chickens tested for the presence of antibodies to CLE by immunodiffusion disclosed that they were also positive in several other of the serological assays (Table III). Only 12 out of 18 line 200 chickens positive for anti-CLE antibodies were used in this correlation analysis because of the availability of sufficient serum for further testing. As shown in Table III, of 12 line 200 chickens positive for precipitating antibodies to CLE, 100% also had high anti-ssDNA ELISA values (>2 SD from the control mean), and 100% had positive IIF tests, generally of a fine speckled pattern with some cytoplasmic staining. It was also found that all presented with 100% comb involution, the major early clinical marker of disease expressions. To put these findings in perspective, only 40.4% of line 200 chickens had ssDNA ELISA values > 2 SD from the line 010 mean, 67.7% of line 200 had positive IIF tests (this screening for ANA and ACA was separate from the study earlier reported) and 74.1% of line 200 presented with total comb loss. Of those line 200 with negative immunodiffusion tests to CLE,

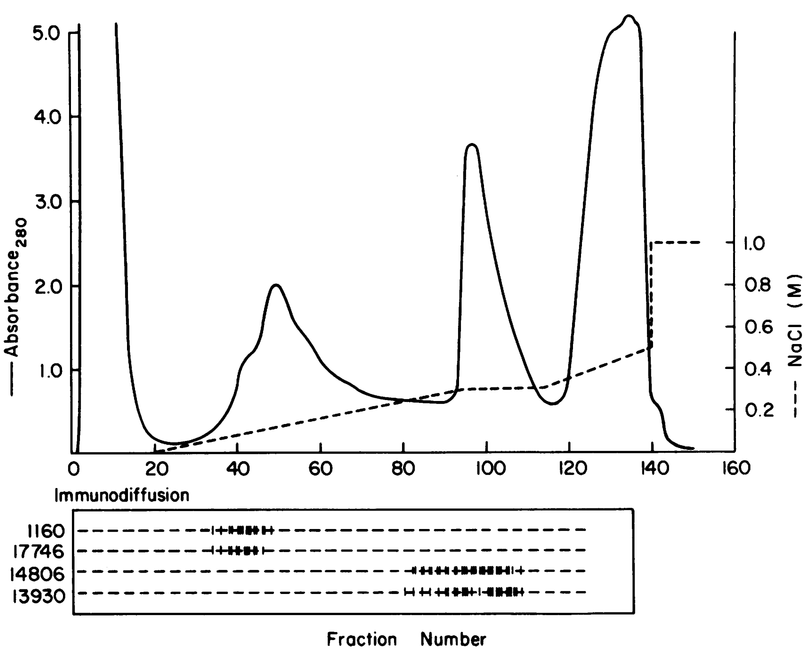

Figure 8. DEAE-Sephacel chromatography of CLE. Antigenic activity, as examined by immunodiffusion testing with line 200 sera, was present in two peaks at NaCl concentrations of ~0.1 and 0.3 M.

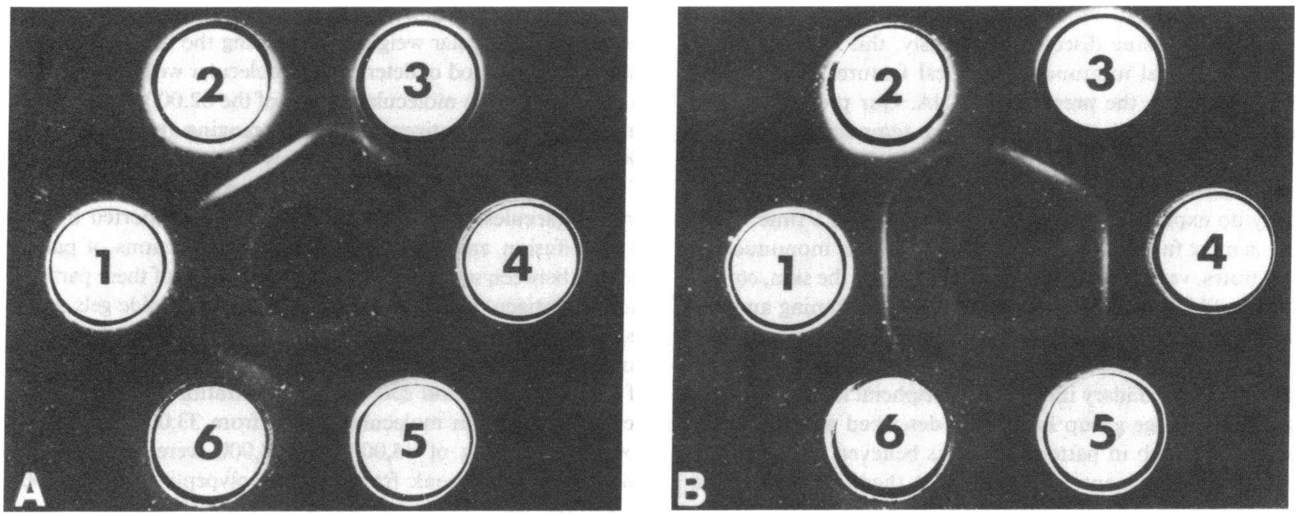

Figure 9. Immunodiffusion of line 200 sera against CLE purified by ion-exchange chromatography with DEAE-Sephacel followed by gel filtration on Sephacryl S-300. Outer wells contain line 200 immunoglobulin fractions from bird 13930 (*1*), 1160 (*2*), 14313 (*3*), 14806 (*4*), 14827 (*5*), and 14583 (*6*). Center wells in *A* and *B* contain partially purified antigens of 58,000 (0.3 mg/ml) and 250,000 (0.5 mg/ml) mol wt, respectively.

only 40% had positive IIF tests. Thus, line 200 chickens that have precipitating antibodies to CLE appear to represent a more severely affected subgroup; however, one must use caution in correlating autoantibodies and disease expression since large numbers and longitudinal studies are necessary for statistically significant conclusions. In contrast, because only 3 out of 14 line 206 sera were found to have anti-CLE activity, no further clinical correlates were studied with this outcross of line 200.

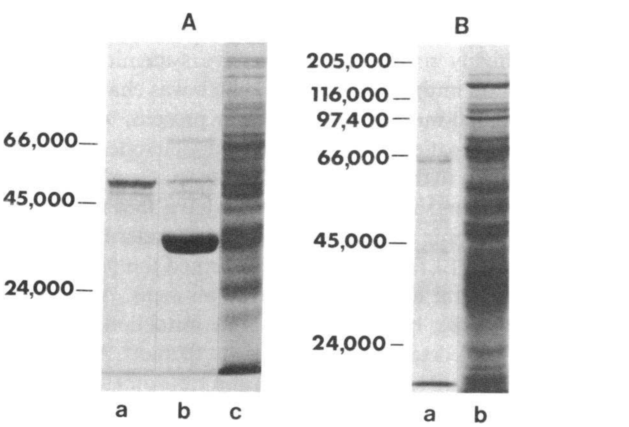

Figure 10. SDS-PAGE of CLE antigens. 12% and 10% slab gels were used in (*A*) and (*B*), respectively. (*A*) CLE was purified by DEAE-Sephacel and then Sephacryl S-300 chromatography. Lanes *a* and *b* contain the 58,000- and 80,000-mol wt Sephacryl S-300 fractions, respectively. Lane *c* contains CLE antigen that has been purified by DEAE-Sephacel chromatography only. (*B*) CLE was purified by DEAE-Sephacel and then Sephacryl S-300 chromatography. Lane *a* contains 250,000-mol wt peak from Sephacryl S-300 fractionation. Lane *b* contains DEAE-Sephacel-fractionated CLE. Molecular weights on the left of *A* and *B* were determined from the relative mobilities of myosin, 205,000; β-galactosidase, 116,000; phosphorylase B, 97,400; BSA, 66,000; ovalbumin, 45,000; and trypsinogen, 24,000.

Discussion

This study was undertaken with two primary objectives; first, to define the nature and characteristics of autoantibodies directed against cellular antigens in line 200 chickens through immunological and physicochemical means and second, to place these

Table III. Correlation between the Presence or Absence of Precipitating Antibodies to Saline-extractable Antigens in Chicken Liver and Other Parameters of Disease Expression in Line 200 Chickens

	Anti-ssDNA ELISA	IIF	100% Comb loss
Positive CLE (n = 12)	100% (12/12)	100% (12/12)	100% (12/12)
Negative CLE (n = 12)	42% (7/12)	50% (6/12)	58% (7/12)

Precipitating antibodies to CLE were assayed by immunodiffusion in line 200 sera. A positive anti-ssDNA ELISA test was taken as a response > 2 SD from the line 010 mean. Indirect immunofluorescence on HEp-2 substrate revealed a mean score of 2.1 for the positive CLE group and a score of 0.5 for the negative CLE group, based on a one to four scoring system. Comb loss was determined by visual observation of line 200 birds up to 2 mo of age.

findings in perspective with what is currently known about human connective tissue diseases. Previously, this laboratory (1) reported on several immunopathological features of line 200, among these being the presence of ANA. Our present studies extend these observations to include the temporal occurrence of not only ANA, but also ACA. Few line 200 chickens are found to have developed ANA as of 1 mo of age (7%), but the majority do express ACA (64.3%). This comes at a time when disease is most fulminant, as indicated by intense mononuclear cell infiltrates, vascular occlusion, and fibrosis in the skin, comb, and digits. This results in comb involution, thickening and necrosis of skin, and foot lesions accompanied by distal polyarthritis. Mortality at this age is 20% (vs. 0% mortality of controls) possibly due to secondary infection of peripheral lesions. Positive IIF tests in this age group have been described as being cytoplasmic spider web in pattern, which is believed to be specific for cytoskeletal elements. The role that these autoantibodies might play in the line 200 disease process is not known at this time, although it is interesting to note that antigenic specificity changes over the course of disease expression. In contrast to the cytoplasmic spider web staining pattern, which is primarily limited to young birds with acute signs of disease expression, other staining patterns (diffuse fine speckles, peripheral, and homogeneous) occur in line 200 chickens that have recovered from the acute onset of disease and evolve into a chronic multisystem organ involvement (1). Studies of line 200 pathology in these older age groups have shown that internal symptoms include vascular occlusion, cellular infiltrates, and fibrosis of skin, heart, lung, and kidney.

Autoantibodies in line 200 sera against ENA are not detected when using rabbit, rat, or mouse sources of tissues. However, saline extracts from chicken thymus and liver were found to be highly antigenic for line 200 sera. Additional testing revealed that antigenicity was also present in the liver of other avian species including the turkey, pheasant, and partridge. Moreover, upon cellular fractionation by differential centrifugation it was determined that these antigens lie within the cytoplasmic compartment. In light of the presence of ANA in line 200 sera it would appear that different antigens are being recognized on the HEp-2 substrate or that these antigens are not saline extractable from chicken liver nuclei. This conclusion was further substantiated by the inhibition of immunofluorescent staining with rabbit thymus powder-absorbed line 200 sera. In comparison, absorption with CLE does not inhibit line 200 fine speckled ANA.

The antigenic determinants of CLE are composed of protein and are not affected by RNase or DNase, although this does not exclude the possibility that RNA, which is soluble under these extraction conditions, could be associated with some of these antigens. Recent studies have shown that the cytoplasmic antigens Ro and La, as well as the nuclear antigens RNP and Sm, contain associated RNA (17). For most sera that recognize these small cytoplasmic or small nuclear RNA-protein particles the antigenic determinants are constituted at least in part of protein. Purification of CLE antigens by ion-exchange and gel-filtration chromatography has demonstrated several antigens on the basis of molecular weight. Considering the inherent imprecision of this method of determining molecular weights (as noted by discrepancies in molecular weight of the 62,000-mol wt CLE antigen), the five antigenic fractions ranging from 62,000 to 290,000 mol wt could quite conceivably represent alternate forms of several smaller polypeptides, which can associate to produce larger molecules. This conclusion is further supported by immunodiffusion analysis, which has shown reactions of partial identity between some of these antigens. Studies of these partially purified antigen preparations on SDS-polyacrylamide gels under reducing and denaturing conditions have shown that partially purified antigenic fractions with approximate molecular weights of 58,000, 80,000, and 250,000 by gel filtration contained polypeptides ranging in molecular weight from 33,000 to 136,000. Two polypeptides of 45,000 and 48,000 were found in the 58,000-mol wt antigenic fraction, four polypeptides from 33,000 to 48,000, with the major polypeptide at 33,000, were found in the 80,000-mol wt fraction and lastly, at least five polypeptides of 38,000, 45,000, 63,000, 96,000, and 136,000 were found in the 250,000-mol wt fraction. At present, it has not yet been determined which of these polypeptides contain the antigenic determinants that line 200 sera recognize.

Characterization of individual antibody-antigen reactions in connective tissue disease states has advanced to where certain antigens are now recognized as specific diagnostic markers (2, 5, 18–22). High titers of antibodies to n-RNP, a complex of RNA and protein, is often associated with mixed connective tissue disease (18). Sm, a protein antigen, is found only in SLE (2) while a 70,000-mol wt nuclear protein, Scl-70, has been found to be highly specific for progressive systemic sclerosis (23). Recently, an antigen termed Ku, which was characterized as being a 300,000-mol wt acidic nuclear protein, was found to be a marker for patients with polymyositis-scleroderma overlap (5). In addition to these and several other "nuclear" antigens isolated from various sources of ENA, there have been numerous reports of cytoplasmic antigens being grouped under ENAs (22, 24–27). Studies of SLE sera have demonstrated the presence of antibodies to several unique cytoplasmic antigens. Antibodies to microsomal (28), lysosomal (29), and mitochondrial (30) antigens have been detected by complement fixation. Antibodies to ribosomal (r) RNP (22), r-RNA (31) and ribosomes (32) have also been found in SLE sera. The cytoplasmic antigens SS-A (Ro) and SS-B (La or Ha) are found in association with SLE and sicca complex (25, 29).

Detailed analysis of RNP, Sm, Ro, and La antigens has shown the interrelationship between these determinants (17, 33–35). They are all found on ribonucleoprotein complexes of either cytoplasmic or nuclear origin; furthermore, they contain several small RNA species and as yet, a controversial number of polypeptides. Studies on immunoprecipitation of RNP and Sm by Conner et al. (33) have shown that RNP contains two polypeptides with molecular weights of 19,000 and depending on the antigen source, 68,000 or 70,000. The Sm antigen contained polypeptides of 25,000 and 16,000 mol wt. Takano et al. (34) has reported that RNP contains six polypeptides; two of them at 13,000 mol wt were antigenic. Molecular weights of the other four polypeptides were 13,000, 13,000, 30,000, and 65,000. In contrast, Gibbons et al. (35) demonstrated that RNP was composed of five polypeptides ranging in molecular weight from 10,000 to 15,000, whereas Sm contained similar proteins plus six additional subunits from 21,000 to 42,000 mol wt. These studies have also been complicated by the finding of variations in composition due to the source of antigens (33). Therefore, comparing the line 200 data with previously published studies is difficult. Our observations suggest that line 200 antigenic specificities are unique on the basis of size and antigenic source. Although some variation has been noted in the literature as to the sources of ENA, most have been found to be highly conserved (6). Repeated attempts to identify the line 200 antigenic specificities in nonavian tissues sources have failed. Our results suggest that line 200 chickens recognize a heterogeneous group of antigens, highly conserved antigens as noted by positive ANA on HEp-2 cells and some not so highly conserved antigens, demonstrated by precipitin reactions with avian liver saline extracts.

Further analysis of the antigen binding diversity of line 200 and line 200 outcrosses was performed by ELISA to ssDNA, histones, RNA, and poly A · poly U. Line 200 sera were nonreactive towards these latter three antigens; however, there were significantly greater numbers of high responders to ssDNA in line 200 than in control line 010 (40.4% vs. 0.0%). Anti-ssDNA responses noted for line 211, (F_1 generation birds from line 200 × line 011) and line 206 (produced by matings involving line 211 several generations removed) suggest that this trait is inherited as an autosomal recessive. Previous genetic studies on line 200 clinical disease expression have also shown that the mode of inheritance is autosomal recessive although there appears to be incomplete penetrance (1).

Line 200 anti-ssDNA reactivities were most sensitive to inhibition by ssDNA, followed by poly G or RNA and then nDNA. Studies of anti-polynucleotide antibodies in sera from patients with connective tissue diseases have shown that there are several antigenic determinants that can be recognized on ssDNA (36, 37) and dsDNA (38). Even within a single disease state such as SLE, there is much individual patient variation as to the determinants recognized on polynucleotides; antigen binding reactivities can be specific for the sugar phosphate backbone (10) or for specific bases (39). The lack of inhibition by phospholipids with line 200 sera suggests that these autoantibodies do not recognize the sugar phosphate backbone. However, inhibition by poly G could be due to base specificity. Further analysis of the line 200 anti-ssDNA response might reveal a heterogeneous population of antibodies directed toward ssDNA as they do toward other cellular antigens.

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