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CONCENTRATION OF AUTOANTIBODIES TO NATIVE 60-kd Ro/SS-A AND DENATURED 52-kd Ro/SS-A IN ELUATES FROM THE HEART OF A CHILD WHO DIED WITH CONGENITAL COMPLETE HEART BLOCK

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Objective. **To determine the serologic specificity of acid eluates from tissues of a child who died with congenital complete heart block (CCHB).**

Methods. **Tissues were extracted, acid eluted, and the IgG and antibody titers determined on the eluates by enzyme-linked immunosorbent assay.**

Results. **Antibodies to native 60-kd and denatured 52-kd Ro/SS-A were found to be enriched only in the heart eluate, and not in the eluates from brain, kidney, and skin.**

Conclusion. **These findings indicate a major role for anti–native 60-kd Ro/SS-A in the immunopathogenesis of CCHB.**

Neonatal lupus erythematosus is a disease that is characterized frequently by dermatitis and congenital complete heart block (CCHB), less commonly by thrombocytopenia, and rarely by cholestatic hepatitis. The disease is usually associated with transplacental passage of IgG antibodies to the small ribonucleoprotein particle Ro/SS-A and, slightly less frequently, La/SS-B (1–9). Rarely, neonatal lupus dermatitis is associated with antibodies to the ribonucleoprotein particle U1 RNP (10).

While recent studies have attempted to assess risk of a mother with systemic lupus erythematosus (SLE) or an asymptomatic mother with antibodies to Ro/SS-A and/or La/SS-B bearing a child with CCHB, this is at best an indirect approach to risk assessment since the condition develops in only a small fraction of children born to such mothers. Moreover, in the only prospective study to date, of 38 children born to SLE mothers with anti-Ro and/or anti-La or with anti-RNP/Sm, none had CCHB (11). Thus, perhaps only 2–5% of mothers at risk for having a child with CCHB actually do so.

Attention has therefore turned to the study of the hearts of children with CCHB who have died of cardiac failure. In 3 instances, IgG has been demonstrated in the inflammatory deposits in the heart of children with CCHB (12–14). In 1 of these cases, IgG and IgA globulin were found in a diffuse pattern (12), and diffuse cytoplasmic deposits of IgG and IgM were described in another (13). The third report described particulate deposits of IgG, with no IgM or IgA deposition (14). Finally, in a study using anti–La/SS-B antiidiotypic serum, the idiotype of an anti–La/SS-B antibody was seen on the surface of myocardial fibers from a child with CCHB (15). In addition, anti–La/SS-B was demonstrated in both phosphate buffered saline (PBS) washes and guanidinium eluates of heart sections (15). The methods used in the first studies (12–14) do not enable determination of the specificity of the Ig deposits and, like the methods in the anti-idiotype studies (15), are not quantitative.

We report the first quantitative elution studies of several tissues from a child who died in utero with CCHB. The mother had SLE with anti–Ro/SS-A antibodies but no detectable antibodies to La/SS-B or

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other RNA proteins such as U1 RNP or Sm. We determined both the IgG content and the anti–Ro/SS-A activity in the acid eluates of saline-washed homogenates of various organs, as well as in the mother's serum. This enabled us to compare the antibody content per unit weight of IgG in such eluates and to compare these values with the antibody content per unit weight of IgG in the mother's serum. The results showed that autoantibodies to the native 60-kd Ro/SS-A molecule were enriched 2.9-fold in the eluate from the heart tissue compared with the maternal serum, but similarly prepared eluates from the skin, kidney, and brain were not enriched for anti–Ro/SS-A.

PATIENT AND METHODS

Description of the mother with SLE and the child with CCHB. The patient is a 30-year-old Italian woman. She had experienced arthralgias for many years. She gave birth to a healthy baby in 1989. In 1991 she presented with adenopathy, low-grade fever, and a butterfly rash on the face. The antinuclear antibody titer was 1:1,080 with a homogeneous pattern as seen on HEp-2 cells.

The *Crithidia luciliae* titer was 1:30, indicating significantly positive anti–double-stranded DNA. The anti–single-stranded DNA level was 21% (normal <10%). The only precipitin demonstrated was anti–Ro/SS-A, on calf thymus extract.

In 1992, the patient noted alopecia. During that year she became pregnant for the second time. At 21 weeks of gestation, fetal echocardiography showed a normal fetal heart with a ventricular rate of 50–60 beats per minute, while the atrial rate was 140–150 beats per minute; fetal signs of heart failure were absent. The maternal anti-Ro titer was 1:128. At 34 weeks of gestation, a few days before the scheduled caesarean section, the mother noted decreased spontaneous fetal movements. She was seen by an obstetrician, who performed serial fetal echographies (March 22, 24, and 25, 1993). On March 25, the fetus was found to be dead. The next day, labor was induced, and a girl weighing ~2,600 gm was born. Fetal tissues were frozen immediately, and maternal serum was stored. These were shipped on dry ice to Oklahoma City, by overnight delivery.

Preparation of eluates. Tissues were weighed in the frozen state on a Mettler PC400 pan balance, to within 0.1 gm. The samples, which varied in size from 1.1 gm (skin) to 9.0 gm (heart), were then mixed with 3 volumes of PBS, pH 7.2, and homogenized with either a hand-held homogenizer (skin and kidney) or a small Waring blender (heart and brain). The comparable efficiency of the homogenization methods was demonstrated by the very similar IgG concentrations and anti–Ro/SS-A titers in the saline extracts. Indeed, the anti–Ro/SS-A activity per μg IgG was indistinguishable in the saline extracts for all 4 organs.

The homogenates were spun for 40 minutes at 900*g* in a refrigerated centrifuge. The supernatants were separated (saline extract of tissue), and the pellets were washed 4 times in the cold with 3 volumes of PBS and centrifuged. The final concentration of IgG in the fourth wash was ≤0.2 μg/ml in all cases, as determined by a sandwich assay for Ig determination (see below). The pellets were then extracted with 0.2*M* glycine buffer, pH 2.4, as described previously (16). The acid eluates were dialyzed against 100 volumes of PBS. Precipitates were removed by centrifugation, and the soluble eluates were then studied for IgG concentration, antibody to bovine Ro/SS-A by enzyme-linked immunosorbent assay (ELISA), and antibody to La/SS-B by ELISA. The precipitates were studied by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) for the presence of 60-kd and 52-kd Ro/SS-A as well as for La/SS-B, and none was found. Serum samples from the mother were tested for all these parameters simultaneously on the same ELISA plates.

Analytic immunochemical methods. ELISA for antibody to Ro/SS-A and La/SS-B was performed as described previously (17). All experiments were performed in duplicate, and 2 independent preparations beginning with weighed tissue were used for each of the various tissue sources. Results of the 2 experiments were always indistinguishable.

Immunofluorescence technique. Four micron–thick frozen sections of heart, kidney, and skin were incubated with rabbit $F(ab')_2$ fluorescein-conjugated anti-human IgG, IgM, and IgA (Dako, Carpinteria, CA) at 1:250 dilutions for 2 hours at room temperature. Mounting medium consisted of glycerol-PBS with 0.1% *p*-phenylenediamine.

The ELISA for quantitating IgG was carried out by a sandwich method. Affinity-purified goat anti-human IgG (Sigma, St. Louis, MO) at a concentration of 2 μg/ml in 0.05*M* carbonate buffer, pH 9.2, was coated onto polyvinyl chloride microtiter plates (Costar, Cambridge, MA). A standard curve was constructed with concentrations of Cohn fraction II from 2.0 μg/ml to 0.02 μg/ml. Dilutions of serum and eluates were used to charge plates, which were always accompanied by a standard curve. Binding of IgG was assessed by development with a goat anti-human IgG (γ chain–specific) alkaline phosphatase conjugate (affinity purified; Sigma) at a dilution of 1:5,000, followed by the substrate NBT/BCIP. Color development was read at 405 nm with a Dynatech (Alexandria, VA) scanner. IgG and anti–Ro/SS-A ELISAs were always carried out with precisely the same diluted samples of serum and eluate on the same plate.

ELISA for antibodies to recombinant 52-kd Ro/SS-A. An ELISA was developed to detect antibodies that bind to the human recombinant 52-kd Ro protein expressed in *Escherichia coli* under the control of the *lac* promoter. The protein was solubilized in 7*M* urea after lysis of the cells by sonication. These proteins were diluted in a carbonate buffer (pH 9.6, with 1 m*M* $MgCl_2$) and used to coat 96-well plates (Costar, Cambridge, MA). Optical densities (OD) at 405 nm were detected after reaction with an alkaline phosphatase–conjugated goat anti-human IgG (Sigma) and 1 mg/ml *p*-nitrophenyl phosphate. These values were corrected by subtracting optical densities obtained from similarly treated extracts from *E coli* that contained the expression vector but lacked the human 52-kd Ro complementary DNA insert.

Table 1. Yield of acid-eluted IgG from various organs of the stillborn child with congenital complete heart block

Organ	Eluted IgG, μg/gm tissue
Heart	15.7
Skin	3.7
Kidney	4.0
Brain	3.0

RESULTS

Quantitation of eluate immunoglobulin from the various organs is listed in Table 1. It is noteworthy that the heart yielded, on average, ~4 times as much IgG in the neutralized acid eluates as did skin, kidney, or brain. In parallel with this finding, the only eluate whose specific activity differed from that of the maternal serum (OD in anti–Ro/SS-A ELISA/mg IgG) was the heart eluate (Figure 1).

As seen in Figure 1, the curve for the heart eluate was displaced to the left, meaning that this IgG was more active as anti-Ro on a weight basis, compared with maternal serum IgG or the eluates from the other tissues. By comparing the quantities of IgG required to achieve the same OD along the curves of the serum and the heart eluate, the ratio of serum IgG: eluate IgG was calculated to be 2.9. This can be considered to be an enrichment ratio, meaning that eluate IgG was 2.9 times more effective than serum IgG in anti–Ro/SS-A activity. This is taken as evidence

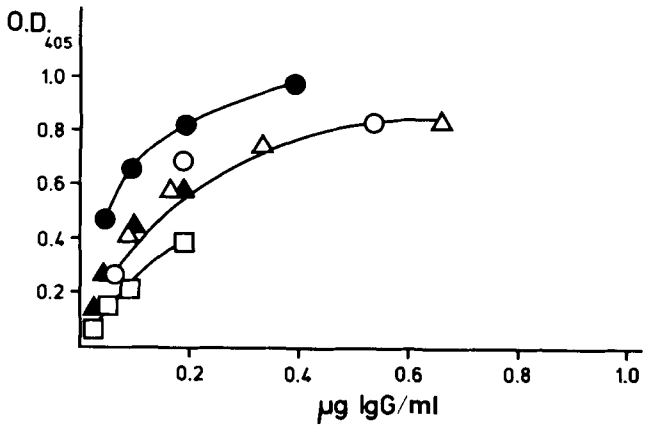

Figure 1. Results of enzyme-linked immunosorbent assay on polyvinyl chloride microtiter plates coated with bovine native 60-kd Ro/SS-A and then tested with serum from the patient (○) and eluates from various tissues, including heart (●), brain (△), kidney (▲), and skin (□), from her stillborn child with congenital complete heart block. $O.D._{405}$ = optical density at 405 nm.

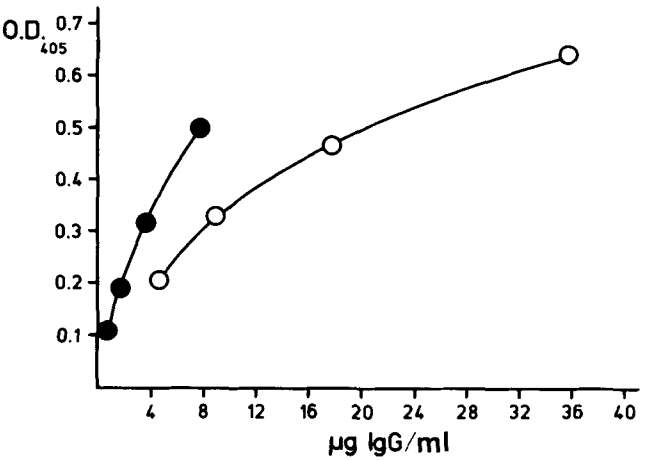

Figure 2. Results of enzyme-linked immunosorbent assay on polyvinyl chloride microtiter plates coated with recombinant 52-kd Ro/SS-A and then tested with serum from the patient (○) and heart eluate from her stillborn child with congenital complete heart block (●). $O.D._{405}$ = optical density at 405 nm.

for specific deposition of autoantibody to the Ro/SS-A antigen in the heart. As seen, the only curves displaced from the serum were those for the heart eluate and the skin eluate. In contrast to the heart eluate, which showed enrichment of anti–Ro/SS-A activity, the skin eluate curve was displaced slightly to the right, suggesting a loss of specific activity. This difference could also be nonsignificant.

Figure 2 shows the curves generated with the heart eluate and the maternal serum in an ELISA that was specific for antibody to recombinant 52-kd Ro/SS-A. As seen, the curve for the heart eluate was displaced to the left of the serum curve, and the enrichment ratio (μg serum IgG:eluate IgG, which gave identical OD in ELISA) was 2.6, very similar to that achieved in the anti–60-kd Ro/SS-A ELISA.

In this assay, 125–144 times more IgG was required to achieve the same OD in the anti–52-kd Ro/SS-A ELISA as in the anti–60-kd Ro/SS-A ELISA. This is consistent with the recent findings that antibodies to the denatured form of 52-kd Ro/SS-A (measured with recombinant 52-kd Ro/SS-A or Western blot) are a subset of antibodies to native 60-kd Ro/SS-A that cross-react with 52-kd Ro/SS-A (18). If findings in the assays were linear reflections of the antibody concentrations, then the anti–52-kd Ro/SS-A antibody response would be ~0.75% of the anti–60-kd Ro/SS-A response. Whether the anti–52-kd Ro/SS-A response is 1% or 10% of the anti–60-kd Ro/SS-A response, the fact that the enrichment ratio for the antibody to the

52-kd Ro/SS-A protein in the eluate (2.6) was very similar to that for the anti–60-kd Ro/SS-A in the eluate (2.9) is evidence that there was no further enrichment of the antibody to the 52-kd Ro/SS-A. Thus, the major antibody (≥90%) to Ro/SS-A concentrated in the heart is that reactive with the native 60-kd Ro/SS-A antigen.

Because the serum was so much less active in response to the recombinant 52-kd Ro/SS-A compared with the native 60-kd Ro/SS-A, only the serum and the heart eluate had measurable activity. No antibody response to the recombinant 52-kd Ro/SS-A was demonstrated in the skin, kidney, or brain eluates. This was not surprising since there was no enrichment for anti–60-kd Ro/SS-A in any of these eluates and the yield of IgG in these cases was ~25% of that found in the heart eluate (see Table 1).

Finally, to ensure the specificity of the heart eluate, the tissue pellet was washed not only 4 times, but, in a second experiment, 7 times before acid elution. While the yield of IgG eluted from the heart dropped by 60%, the specific activity of the eluate after 7 washings was identical to that of the eluate recovered after 4 saline washings (enriched 2.9-fold), as seen in Figure 3. Studies of the IgG content of the washings showed that for the heart, after 3 washings, all subsequent washings had 0.2 μg IgG/ml, which was ~1% of the content of the first wash. Similar results were achieved with the other organs studied.

Finally, immunofluorescence studies were carried out on cryostat sections of heart, kidney, and skin. The heart, kidney, and skin had deposits of IgG, but not IgM or IgA. The IgG deposits were present in all sections of tissue examined and appeared to spare the cell nuclei. In the kidney and skin, the IgG was present in a fine particulate pattern. In the heart, the IgG deposits appeared as larger globules, in comparison with the deposits in kidney and skin.

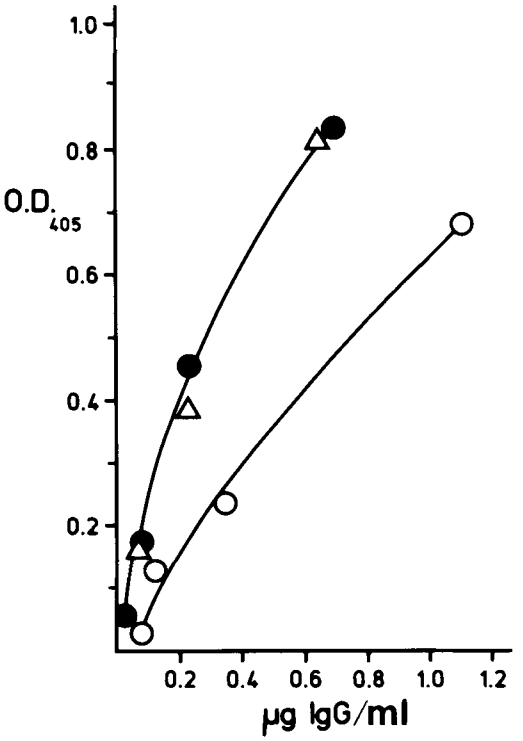

Figure 3. Comparison of results of enzyme-linked immunosorbent assays for antibody to native 60-kd Ro/SS-A in serum from the patient (○), heart eluate 1 from her stillborn child with congenital complete heart block, which was acid eluted after 4 saline washes (●), heart eluate 2 from the child, which was acid eluted after 7 saline washes (△). $O.D._{405}$ = optical density at 405 nm.

DISCUSSION

In these studies, the specific immunologic activity for antibodies to Ro/SS-A antigen in neutralized acid eluates of tissues from a child who died with CCHB was determined quantitatively. The specific enrichment of antibodies to the native 60-kd Ro/SS-A antigen in the neutralized eluates of the heart but not the skin, kidney, or brain provides evidence for the specific deposition of antibodies to the native 60-kd Ro/SS-A molecule. Antibodies to the La/SS-B antigen were undetectable in studies of the serum and eluates, and autoantibodies to the recombinant 52-kd Ro/SS-A antigen, which were enriched similarly to the anti–60-kd Ro/SS-A response, were ≤1% of the antibody in both the serum and the eluate. No studies of antibody response to the native 52-kd Ro/SS-A antigen were performed, since it has been shown in 17 of 17 previously studied anti–Ro/SS-A–positive sera that all of the antibody response to the 52-kd Ro/SS-A antigen is to the denatured form measurable in a recombinant ELISA (19), as was done here.

Such studies rest on the assumption that acid elution of saline-washed homogenates releases specific immune complexes from tissues. After neutralization, any antigen not destroyed by acid would reprecipitate some of the extracted specific antibody, and indeed, precipitates regularly formed after neutralization in these studies. Redissolving such precipitates in the SDS–2-mercaptoethanol buffer used in SDS-PAGE failed to reveal Ro/SS-A antigen when such gels were probed with appropriate sera. We have no simple way to quantitate "anti–Ro/SS-A" in such precipitates,

and so we measured the free anti–Ro/SS-A remaining in acid eluates after neutralization, probably representing only a fraction of the immune complexes actually deposited in the tissue. However, this method, pioneered by investigators at Rockefeller University (20) in the study of acid eluates from the kidneys of patients with lupus nephritis, has been applied to studies of anti–Ro/SS-A in the kidneys of such patients (16) and from the salivary glands of a patient with Sjögren's syndrome (21). These studies all provided strong evidence for deposition of specific antibodies in damaged organs and have been central in the formulation of concepts of immunopathogenesis in these disorders.

This is the first application of such methods to the heart disease of neonatal lupus and provides strong evidence, in a single case, for the involvement of antibodies to the native 60-kd Ro/SS-A antigen in the immunopathogenesis of CCHB. These studies do not rule out the involvement of antibodies to La/SS-B and/or the 52-kd Ro/SS-A in other cases of CCHB. Anti–La/SS-B and antibodies to the 52-kd Ro/SS-A in maternal serum conferred a high risk for CCHB in one study (22), but in another study, no evidence was found for a unique heart block–associated anti–Ro/SS-A autoantibody profile (23). Moreover, in our studies using gel diffusion, ELISA, and Western blot, while all mothers of children with CCHB (11 of 11) had anti–native 60-kd Ro/SS-A, only 4 of 11 CCHB sera tested had anti–La/SS-B by gel diffusion or immunoblotting (24). There may also be other, as-yet-unrecognized, antigen–antibody systems involved in the pathogenesis of this and other cases of neonatal lupus. These studies show that at least one antibody known to be present in the vast majority of mothers, if not all mothers, bearing children with CCHB can specifically concentrate in the heart of the child.

These studies do not answer the question of why CCHB occurs in only a small number of children born to mothers who have anti–Ro/SS-A in their sera, or why adults who carry anti–Ro/SS-A in their sera are so rarely found to have heart block. However, the existence of several fraternal twin pairs discordant for CCHB indicates the presence of an as-yet-unidentified crucial factor in the child (11,25,26). The existence of identical HLA genes in the discordant twin pair for CCHB reported from our laboratory (26) indicates that the factor in the child, if genetic, is not located in the major histocompatibility complex region of chromosome 6. Finally, it is well known that the dermatitis and CCHB tend to occur independently in neonatal lupus erythematosus, making it likely that there are distinctive and independent determinants for these 2 clinical manifestations. Quantitative elution studies of larger numbers of tissues from children who die of CCHB-related heart disease should shed further light on this disorder.

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