Antiagalactosyl IgG Antibodies in Juvenile Idiopathic Arthritis, Juvenile Onset Sjögren's Syndrome, and Healthy Children

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ABSTRACT. ***Objective.*** To determine the normal range of antiagalactosyl IgG antibodies in healthy children, and to investigate the utility of determination of antiagalactosyl IgG antibodies in patients with juvenile idiopathic arthritis (JIA) and juvenile onset Sjögren's syndrome (SS).

Methods. Serum concentrations of antiagalactosyl IgG antibodies were measured in 225 healthy children, 68 patients with JIA (systemic arthritis in 21, polyarthritis in 29, oligoarthritis in 18), and 15 patients with juvenile onset SS, using a lectin-enzyme immunoassay employing prepared human agalactosyl IgG as antigen. A comparison was made between the prevalence and utility of antiagalactosyl IgG antibodies in patients and those of conventional rheumatoid factors (RF) determined by laser nephelometry.

Results. The average serum concentration of antiagalactosyl IgG antibodies for healthy controls was 2.41 ± 0.93 arbitrary units (AU)/ml, and the cutoff value of the normal range was set at 4.3 AU/ml (mean + 2 SD). As a result, antiagalactosyl IgG antibodies were positive in 25 (37%) of 68 patients with JIA, and 14 (93%) of 15 patients with juvenile onset SS, in whom values were much higher than the frequencies of RF positivity. The serum concentrations of antiagalactosyl IgG antibodies in patients were closely correlated with those of RF. Thirteen patients with JIA and 6 patients with juvenile onset SS were positive for antiagalactosyl IgG antibodies despite being negative for RF. With regard to prognosis during followup periods of at least 5 years, JIA patients positive for antiagalactosyl IgG antibodies, even if negative for RF, were resistant to treatment. However, positivity for antiagalactosyl IgG antibodies had no relation to joint destruction.

Conclusion. Our data suggest that antiagalactosyl IgG antibodies, compared with RF, show higher sensitivity to detect immunological disorders in JIA and juvenile onset SS. (J Rheumatol 2004; 31:1211–7)

Key Indexing Terms:
ANTIAGALACTOSYL IgG ANTIBODIES RHEUMATOID FACTORS CHILDREN
JUVENILE IDIOPATHIC ARTHRITIS SJÖGREN'S SYNDROME

Juvenile idiopathic arthritis (JIA) is a chronic inflammatory rheumatic disease, the precise etiology of which remains unclear. The prognosis of JIA differs among the various subtypes[1,2], and the heterogeneous manifestation of JIA has been described[3]. Recently, a new criterion for the classification of the 7 types of childhood arthritis was proposed by the International League of Associations for Rheumatology (ILAR)[4]. Rheumatoid factors (RF) are highly discriminating for 3 of the ILAR categories: polyarthritis-type JIA is classified into 2 types according to RF positivity, and RF positive oligoarthritis is excluded from oligoarticular type. The incidence of RF positivity is indeed low in children with JIA, but it has been reported that patients with JIA who express RF may represent a distinct subset of children prone to severe episodes of synovitis and joint erosion[5-8]. Therefore, it is useful to know the RF status when we treat patients with JIA. In patients with juvenile onset Sjögren's syndrome (SS), compared with those with adult SS, a high frequency of extraglandular manifestations, and immunological abnormalities including positivity for anti-alpha-fodrin antibody, antinuclear antibodies, and RF, despite a lower frequency of subjective symptoms of dryness, is characteristic[9-11]. Indeed, concentrations of RF were reported to correlate to the number of extraglandular manifestations in patients with SS[12]. Thus, RF are considered to be useful for diagnosis of juvenile onset SS.

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RF are autoantibodies directed to the Fc portion of human IgG, and glycosidic chains in the CH2 domain of the IgG-Fc portion are the likely candidates for the epitopes for binding to RF[13]. Recent analytical research on the structure of the glycosidic chains has revealed that serum IgG is eminently deficient in galactose in patients with rheumatoid arthritis (RA), compared to healthy individuals[14,15]. The deficiency of galactose in the CH2 domain changes the 3-dimensional structure of the Fc portion, and that portion's antigenicity is upregulated[13]. The possibility has been reported that this glycosidic chain abnormality, agalactosyl IgG, is associated with the development of RA or RF. Indeed, an increased serum concentration of agalactosyl IgG has also been observed in patients with JIA[16]. Recently, a novel lectin-enzyme immunoassay that detects antiagalactosyl IgG antibodies was developed[17], the Eitest CA-RF from Eisai Co. Ltd. (Tokyo, Japan). This assay kit uses agalactosyl IgG prepared by enzyme treatment as a reactant to antiagalactosyl IgG antibodies, and uses lectin to detect terminal galactose moieties in sugar chains of antiagalactosyl IgG antibodies. Measurements based on this new principle can detect autoantibodies regardless of class, since such methods can detect galactose in sugar chains shared by all immunoglobulin classes. Moreover, if agalactosyl IgG prepared by enzyme engineering techniques can mimic the agalactosyl IgG found in the serum of children with rheumatic disease, it may serve as a better reactant for autoantibodies, making possible more specific and more sensitive methods of detecting immunological disorders. The usefulness of determination of antiagalactosyl IgG antibodies using this kit has been demonstrated in studies on RA, SS, and systemic sclerosis in adults[18,19]. However, to our knowledge, no studies have investigated the usefulness of antiagalactosyl IgG antibodies in children. Our aims were to establish the normal range of antiagalactosyl IgG antibodies in healthy children, and to investigate the utility of determination of antiagalactosyl IgG antibodies in patients with JIA and juvenile onset SS.

MATERIALS AND METHODS

Subjects. A total of 308 subjects, including 68 patients with JIA, 15 patients with juvenile onset SS, and 225 healthy children, were enrolled in this study (Table 1). Informed consent was obtained from the parents of all subjects. All JIA patients met the ILAR classification criteria for JIA[4], and this group comprised 21 patients with systemic arthritis onset, 29 with polyarthritis onset including RF positive (10 patients) and RF negative (19 patients), and 18 with oligoarthritis onset. To investigate the utility of RF for classification of JIA, 2 patients with oligoarthritis and positive RF test were included in the oligoarthritis onset subgroup, although the ILAR criteria recommend that such patients be excluded from oligoarticular type[4]. Sera were obtained from these patients when disease was in the active stage; all had joint symptoms, and systemic patients had remittent fever. Active arthritis was defined by the American College of Rheumatology criteria: presence of swelling or, if no swelling is present, limitation of motion accompanied by heat, pain, or tenderness[2]. The extent of joint destruction was evaluated according to staging by the Steinbrocker classification system[20]. Among the patients with juvenile onset SS, 12 had primary SS and 3 had secondary SS (SS associated with systemic lupus erythematosus in 2 patients and with mixed connective tissue disease in one patient). The diagnosis of SS was based on preliminary criteria for the classification of SS[21] with some modifications, as described by Bartunkova, *et al*[22]. Only 3 of the patients with SS had symptoms of dryness. We investigated 225 healthy children (age range 0.5 to 15.8 yrs) in order to set a cutoff point. Most of the control samples were obtained during a medical checkup at school, with parents' informed consent. In addition, some samples were obtained from children who were admitted for selective surgeries such as inguinal herniation or who were undergoing investigation for suspected endocrinological disease. All healthy controls were afebrile, nonarthritic, free of medications, and were shown by examination to be in normal health.

All blood samples were centrifuged at 2000 rpm for 15 min, and sera were stored at –80°C until use.

Measurement of antiagalactosyl IgG antibodies and IgM RF. Serum levels of antiagalactosyl IgG antibodies were determined by a lectin-enzyme immunoassay kit, the Eitest CA-RF (Eisai), using human agalactosyl IgG as the antigen, as described[17-19]. This test kit contains prepared items, such as agalactosyl IgG coated microplates, used in this study. The concept of this assay is illustrated in Figure 1, and details of the assay system are as follows. The agalactosyl IgG was prepared from commercially available human IgG by sugar-chain engineering techniques. In brief, human IgG (10 mg) was treated with 1 U neuraminidase in 0.1 M acetate buffer (pH 5.0) for 24 h at 37°C, followed by treatment with 0.1 U beta-galactosidase in 0.1 M citrate-phosphate buffer (pH 7.0) for 48 h at 37°C. Then the enzyme-treated IgG, agalactosyl IgG, was purified using a protein G coupled to agarose as an affinity column (ImmunoPure Immobilized Protein G; Pierce Biotechnology, Rockford, IL, USA) for chromatography. Equal volume of binding buffer (ImmunoPure IgG binding buffer, pH 5.0; Pierce) was added to the agalactosyl IgG solution, and the mixture was applied to the protein G column (1 × 4.5 cm). After the column had been washed with the same buffer, agalactosyl IgG was eluted with elution buffer (ImmunoPure IgG elution buffer; Pierce), fractionated, and collected into tubes containing 0.1 M phosphate buffer (pH 7.4) to neutralize the pH. Agalactosyl IgG thus obtained was dialyzed against 50 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.01% sodium azide, and kept at 4°C until use. Through structural analysis of sugar chains[14], we confirmed that the prepared agalactosyl IgG completely lacked galactose moieties. The wells of polystyrene microplates (Eisai) were coated with 100 µl of agalactosyl IgG (5 µg/ml) at 4°C overnight. After a washing with Tris-buffered saline (TBS, 0.01 M, pH

Table 1. Characteristics of patients and controls.

Subjects	N	F	M	Age, yrs (range), mean ± SD
JIA				
Systemic arthritis	21	8	13	10.0 ± 4.1 (2.7–14.8)
Polyarthritis	29	24	5	12.6 ± 5.0 (1.5–18.9)
Oligoarthritis	18	13	5	9.6 ± 4.7 (4.0–16.9)
Sjögren's syndrome	15	13	2	13.2 ± 2.9 (7.3–17.6)
Healthy controls	225	109	116	9.3 ± 4.1 (0.5–15.8)

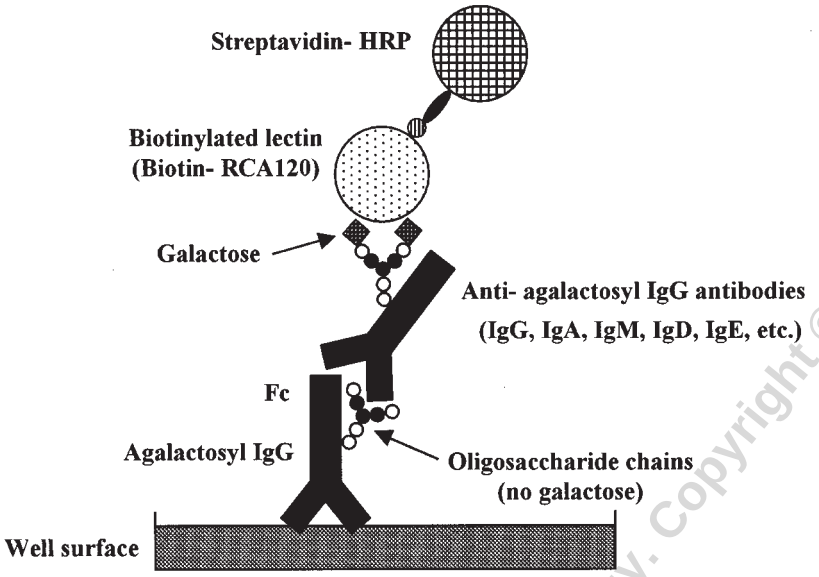

Figure 1. The assay for measuring antiagalactosyl IgG antibodies. Agalactosyl IgG is used as a reactant to antiagalactosyl IgG antibodies, and lectin (RCA 120) is employed to detect terminal galactose moieties in sugar chains of antiagalactosyl IgG antibodies. It was confirmed that RCA 120 did not bind to agalactosyl IgG at all. The antiagalactosyl IgG antibodies are thought to recognize the altered Fc portion of agalactosyl IgG.

7.4), the wells were blocked with 150 µl of TBS containing 0.05% bovine serum albumin (BSA) at 4°C overnight. One hundred microliters of standard solution or serum samples diluted 1:201 with the dilution solution (0.25% BSA, 50 mM Tris-HCl, 0.15 M NaCl, 0.05% polyoxyethylene-octylphenyl ether, 0.02% p-hydroxylbenzoic acid methyl, 0.5% 2-chloroacetamide, pH 7.4) were added to the wells coated with agalactosyl IgG. After 1 h incubation, the wells were washed and 100 µl of biotinylated ricinus communis agglutinin 120 (RCA 120, Seikagaku Corp., Tokyo, Japan) solution was added. Biotinylated RCA 120 detects terminal galatose moieties in antiagalactosyl IgG antibodies. We confirmed that RCA 120 did not bind to agalactosyl IgG at all. After 1 h incubation, the wells were washed and streptavidin-peroxidase conjugate solution (100 µl) was added. After one additional hour of incubation and another washing, 100 µl of chromogen substrate solution was added. The reaction was stopped with 2 mM sodium azide after a 30 min incubation, and the absorbance was read at 405 nm using an ELISA plate reader. The level of antiagalactosyl IgG antibodies in sera was calculated from the calibration curve obtained from the value of standard antibody solution [3.125–50 arbitrary units (AU)/ml]. The reproducibility of this assay, including its repeatability, between-day reproducibility, and interoperator reproducibility, was preliminarily verified[17] in our earlier study. We confirmed that the control serum from healthy subjects showed no absorbance at any dilution ratio. This finding also proved that the agalactosyl IgG fixed on the plate lacked galactose moieties, since the RCA 120 would detect any galactose moieties that might exist in agalactosyl IgG, resulting in some absorbance. In addition, we checked that the probable interfering substances, such as free bilirubin, conjugated bilirubin, hemolytic hemoglobin, and chyle, did not affect the absorbance of the specimens containing specific concentrations of interfering substances[17].

Conventional IgM RF were measured using laser nephelometry (LN), and values > 40 IU/ml were considered positive according to reported values[23,24].

Statistical analysis. Statistical analysis was performed by Mann-Whitney U test for unpaired samples, Scheffe's F test, and Spearman rank correlation coefficient on the Statview program (Abacus Concepts Inc., Berkeley, CA, USA). For all tests, $p < 0.05$ was considered statistically significant. The upper limit of the normal range of antiagalactosyl IgG antibodies was set at the mean + 2 SD of control value, as commonly used as the cutoff values[25-27].

RESULTS

Serum concentrations and frequency of antiagalactosyl IgG antibodies in controls and patients. The average serum concentration of antiagalactosyl IgG antibodies for the 225 healthy controls was 2.41 ± 0.93 AU/ml (mean ± SD) (Figure 2). The upper limit of the normal range was set at 4.3 AU/ml (mean + 2 SD). There were no differences related to age or sex in the controls. The serum concentrations of antiagalactosyl IgG antibodies in patients with JIA and juvenile onset SS are shown in Figure 3. When the cutoff value of antiagalactosyl IgG antibody levels was set as 4.3 AU/ml, antiagalactosyl IgG antibodies were positive in 25 (37%) of 68 patients with JIA, and 14 (93%) of 15 patients with juvenile onset SS. In contrast, the prevalence of RF positivity in patients with JIA and juvenile onset SS were 12 (18%) of 68 and 8 (53%) of 15, respectively. Therefore, the frequencies of antiagalactosyl IgG antibodies in patients with JIA and juvenile onset SS were much higher than those of RF. All patients with RF-positive polyarthritis were also positive for antiagalactosyl IgG antibodies. Further, antiagalactosyl IgG antibodies were positive in 8 of 19 patients with RF negative polyarthritis and in 6 of 18 patients with oligoarthritis. No patient was negative for antiagalactosyl IgG antibodies and positive for RF. Four JIA patients with systemic onset extended to polyarthritis in their course. However, respective antiagalactosyl IgG antibodies in these patients were still negative (data not shown).

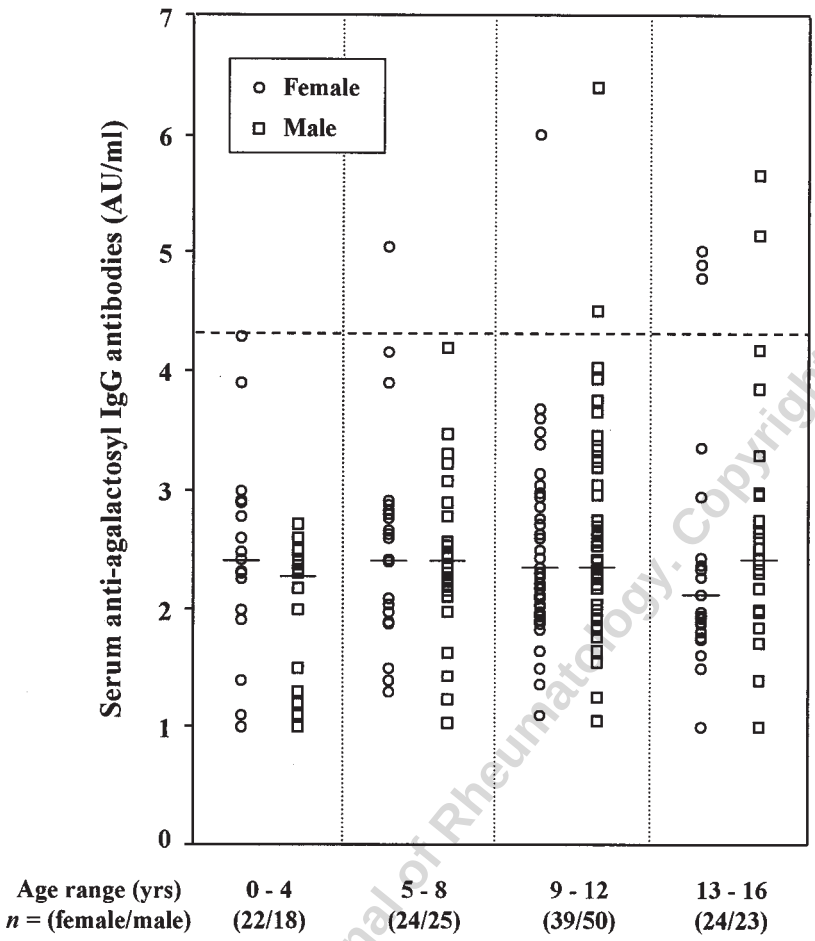

Figure 2. Serum levels of antiagalactosyl IgG antibodies in healthy children. The short solid lines represent the mean value in each group, and the broken line represents the value of mean + 2.0 SD (4.3 AU/ml).

Correlation between antiagalactosyl IgG antibodies and RF in patients. We investigated the correlation between serum levels of antiagalactosyl IgG antibodies and RF. In patients with JIA and juvenile onset SS, serum levels of antiagalactosyl IgG antibodies were significantly correlated with those of RF ($r = 0.79$, $p < 0.0001$ and $r = 0.75$, $p < 0.001$, respectively; Figure 4).

Relation between antiagalactosyl IgG antibodies and prognosis. We compared the utility of antiagalactosyl IgG antibodies with that of RF as a prognostic marker in patients with JIA. First, in 38 patients with JIA (polyarthritis in 25 and oligoarthritis in 13) who were followed for more than 5 years from onset of disease, the relation between positivity for antiagalactosyl IgG antibodies and probability of remission was investigated (Table 2). Among the 19 patients negative for both antiagalactosyl IgG antibodies and RF (double negative), 11 patients went into remission within 5 years. In contrast, no patient among the 8 positive for both antiagalactosyl IgG antibodies and RF (double positive) went into remission during this period. Notably, 10 of the 11 JIA patients who were positive for antiagalactosyl IgG antibodies but negative for RF did not achieve remission during this period. Further, in the same group, we studied the relation of antiagalactosyl IgG antibodies with prognosis for joint destruction (Figure 5). The extent of joint destruction as determined by Steinbrocker staging in the double-positive group was significantly progressive compared with that in patients in the double-negative group. In contrast, statistically, no differences in joint condition were observed between patients who were positive for antiagalactosyl IgG antibodies and negative for RF and patients in the double-negative group.

DISCUSSION

The cutoff value of serum antiagalactosyl IgG antibodies in healthy children was set at 4.3 AU/ml. This value is slightly lower than the cutoff value of healthy adults (6.0 AU/ml) in previous reports[18,19]. Studies with a large number of healthy subjects have demonstrated that serum concentrations of agalactosyl IgG are age-related[15,16,28,29]. Parekh, *et al* showed that total serum agalactosyl IgG decreased from birth to a minimum at 25 years of age, then increased contin-

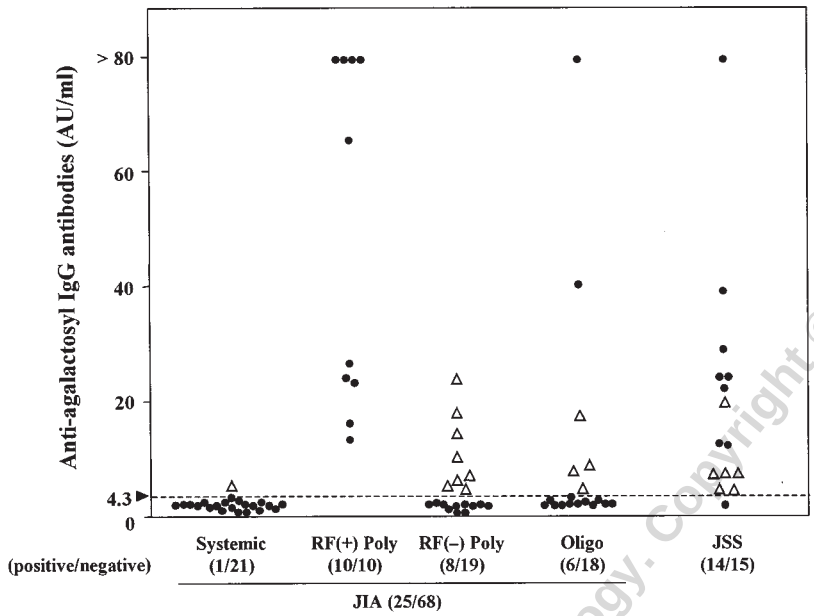

Figure 3. Serum levels of antiagalactosyl IgG antibodies in patients with JIA or juvenile onset SS (JSS). Δ: Patients who were positive for antiagalactosyl IgG antibodies despite being negative for IgM-RF determined by laser nephelometry. Broken line represents the cutoff value (4.3 AU/ml). Systemic: patients with systemic arthritis, Poly: patients with polyarthritis, Oligo: patients with oligoarthritis.

uously with age[28]. Therefore, the cutoff value of antiagalactosyl IgG antibodies should be varied according to age. The cutoff value of antiagalactosyl IgG antibodies in children is expected to be lower than that in adults, although further investigation including analysis of sensitivity and specificity is needed to evaluate the usefulness of our cutoff value.

Antiagalactosyl IgG antibodies were positive in 25 (37%) of 68 patients with JIA in this study. It is noteworthy that 13 patients with RF negative (so-called seronegative) JIA were positive for antiagalactosyl IgG antibodies. Previous reports have described that patients with RA are frequently positive for antiagalactosyl IgG antibodies (77–84%), and that these antibodies are detectable in more than half of all seronegative RA patients[18,30]. The high frequency of antiagalactosyl IgG antibody positivity found in our study in JIA patients, and especially in patients with polyarticular JIA, is in accord with these reports. We also showed that serum concentrations of antiagalactosyl IgG antibodies were closely correlated with those of RF in patients. Combining previous results with the findings in this study, it is suggested that antiagalactosyl IgG antibodies are more sensitive markers reflecting the nature of conventional RF in children. This may be because of the high sensitivity of this assay itself, but also because this method can detect all isotypes of the immunoglobulins. Indeed, it has been reported that almost all patients with RA or SS who had at least one of the RF isotypes (IgG, IgA, or IgM) were also positive for antiagalactosyl IgG antibodies[18].

It is crucial to clarify what this assay is measuring as antiagalactosyl IgG antibodies. Theoretically, all molecules bound to agalactosyl IgG antigen are detected by this assay if they have galactose moieties. We cannot deny the possibility that this assay might detect other RF-unrelated molecules targeting agalactosyl IgG, such as antiidiotype antibodies. In this sense, further fundamental investigations would be required. However, the close correlation between the serum levels of antiagalactosyl IgG antibodies and those of RF suggest that our assay detects rheumatoid factors. Further, we had preliminarily confirmed that our agalactosyl IgG, compared with intact IgG, exhibited significantly higher affinity to purified IgM-RF[17]. From these points, we believe that antiagalactosyl IgG antibodies measured by this assay are more sensitive to RF given the nature of conventional RF.

Among the patients with JIA, antiagalactosyl IgG antibodies were highly detected in polyarticular type (62%), whereas they were detected in only one patient with systemic JIA (5%). These findings may indicate a difference in the pathogenesis between systemic JIA and polyarticular JIA. In general, it is considered that few patients with oligoarticular type JIA show positive RF[2,31], and the ILAR criteria recommend that JIA patients with RF should be excluded from oligoarticular type[4]. However, in our study, 6 of 18 patients with oligoarticular JIA showed positive antiagalactosyl IgG antibodies. Although this result might be due to the high sensitivity of the assay for antiagalactosyl IgG antibodies, additional longterm followup of these patients is required. Further, selection of the assay to determine RF for

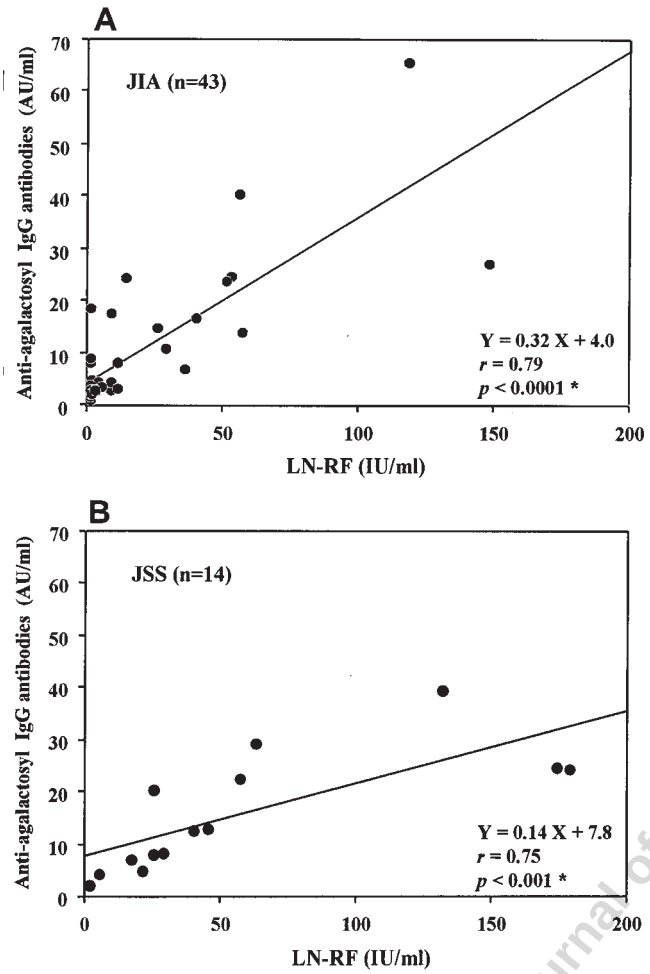

Figure 4. Correlation between serum levels of antiagalactosyl IgG antibodies and RF in patients with JIA (A) or juvenile onset SS (JSS) (B). *Spearmann correlation coefficient. LN-RF: IgM-RF determined by laser nephelometry.

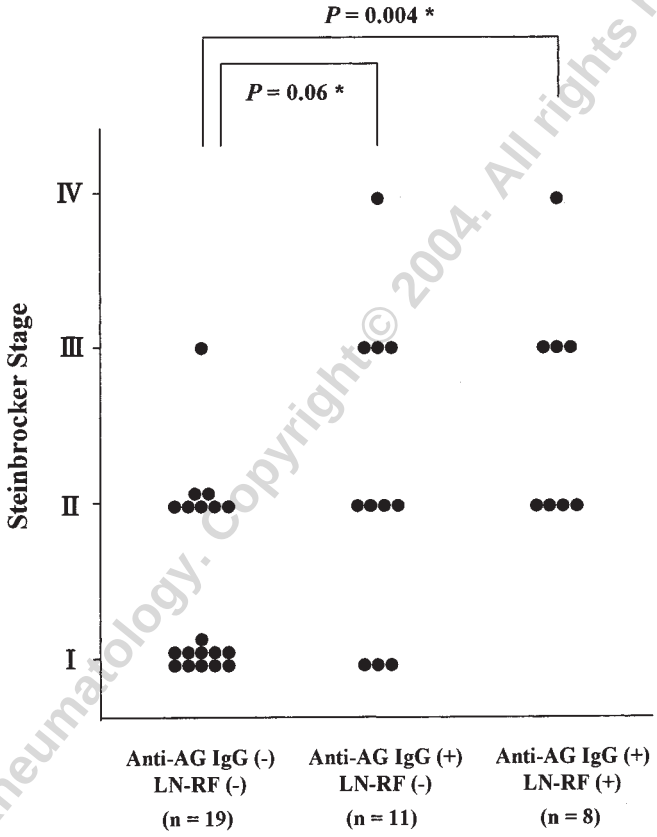

Figure 5. Relationship between joint destruction and RF in 38 patients with polyarticular or oligoarticular JIA followed for more than 5 years. *Scheffe F test. Anti-AG IgG: antiagalactosyl IgG antibodies, LN-RF: IgM-RF determined by laser nephelometry.

purposes of classifying the JIA subtype should be examined. It is an attractive hypothesis that JIA with antiagalactosyl IgG antibodies is identical to RA in adults. However, at present, we do not have enough data to answer this question. To prove this hypothesis, further investigations are required.

With regard to the prognosis, our data showed that the condition of JIA patients who were positive for antiagalactosyl IgG antibodies tended to be intractable regardless of RF positivity. Although no relation was found between positivity for antiagalactosyl IgG antibodies and joint destruction in this study, prolonged disease duration may lead to joint erosion and disability. It is suggested that JIA patients positive for antiagalactosyl IgG antibodies, even if negative for RF, should be monitored closely.

In patients with juvenile onset SS, the positivity of antiagalactosyl IgG antibodies was notably high (93%). A similar high frequency has also been described in adult SS[18]. We should understand that the presence of antiagalactosyl IgG antibodies is not specific for JIA; these antibodies are also frequently present in juvenile onset SS.

Table 2. Correlation between remission and rheumatoid factors in patients with JIA.

	Remission	Nonremission
Anti-AG IgG (–)/RF (–), n = 19	11	8
Anti-AG IgG (–)/RF (+), n = 0	0	0
Anti-AG IgG (+)/RF (–), n = 11	1	10
Anti-AG IgG (+)/RF (+), n = 8	0	8

Anti-AG IgG: antiagalactosyl IgG antibodies, RF: IgM rheumatoid factor determined by laser nephelometry.

Our data raise the possibility that determination of antiagalactosyl IgG antibodies, compared with RF by laser nephelometry, is a more sensitive method to detect RF in patients with JIA or juvenile onset SS. However, at present, the analysis of specificity of antiagalactosyl IgG antibodies is not sufficient. Further investigations accumulating abundant data from patients with JIA are needed to elucidate the utility of antiagalactosyl IgG antibodies as a diagnostic and prognostic marker. In addition, the role of agalactosyl IgG-RF complexes in the pathogenesis of rheumatoid disease is currently under investigation in our laboratory.

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