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Immunology

Agalactosyl glycoforms of IgG autoantibodies are pathogenic

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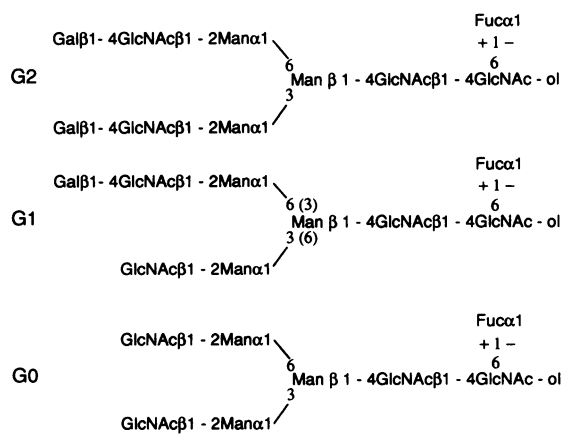
ABSTRACT **The glycosylation of IgG results in many different glycoforms. A large body of correlative data (including remission of arthritis during pregnancy) has suggested that IgG molecules lacking galactose were associated with rheumatoid arthritis. We now demonstrate that agalactosyl IgG glycoforms are directly associated with pathogenicity in murine collagen-induced arthritis. We show that passive transfer of an acute synovitis in T-cell-primed mice can be enhanced by using IgG containing autoantibodies to type II collagen when the antibodies are present as the agalactosyl glycoform. Thus, nonpathogenic doses of autoantibodies can be made pathogenic by altering their glycosylation state.**

We have shown previously that glycosylation of the IgG molecule varies with age (1). In a range of studies that have allowed for this age-dependent variation we have shown that glycosylation of the IgG molecule is altered in patients with rheumatoid arthritis (2–4). It is now well established that increased expression of the agalactosyl IgG glycoforms occurs in patients with adult rheumatoid arthritis (5); juvenile onset arthritis (6); Crohn disease (7); and the mycobacterial diseases tuberculosis (2, 4), sarcoidosis (G. Rook, personal communication), and a form of leprosy called erythema nodosum leprosum (8). IgG isolated from serial bleeds of patients with juvenile onset arthritis who go into spontaneous remission (6) and from adult rheumatoid arthritis patients who are in pregnancy-induced remission (9) has demonstrated that the increased expression of the agalactosyl IgG glycoform is reversible and not a constitutive change.

Agalactosyl IgG is a set of immunoglobulin glycoforms characterized by having two Fc-associated oligosaccharide chains terminating in *N*-acetylglucosamine rather than galactose/sialic acid (5). These oligosaccharide chains are termed G0 (no galactose) in contrast to G1 (one terminal galactose residue) or G2 (two terminal galactose residues). These structures for mouse IgG are as follows:



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Since the Fc portion of each IgG molecule contains two paired carbohydrate chains, various heterologous combinations of G0-, G1-, and G2-type chains are possible (G0–G1, etc.) in addition to homologous pairings (e.g., G1–G1 or G0–G0).

We have recently been able to demonstrate that pregnant DBA/1 mice with type II collagen-induced arthritis (CIA) undergo a pregnancy-induced remission and postpartum relapse, which correlates with changes in serum levels of agalactosyl IgG (9). These studies suggest that CIA in the DBA/1 mouse may be a good model for the glycosylation changes that occur in human patients with rheumatoid arthritis. Human patients with rheumatoid arthritis have synovial B-cell populations that are skewed toward the synthesis of anti-type II collagen IgG (10). Purified anti-type II collagen IgG from the serum of a rheumatoid arthritis patient has been shown to be arthritogenic in the murine passive transfer system (11). These latter observations are consistent with the hypothesis that localized synovial anti-type II collagen autoantibody production in human patients with rheumatoid arthritis may contribute to joint destruction.

Despite the excellent correlation between disease activity and level of agalactosyl IgG in rheumatoid arthritis, it is still unclear whether this form of IgG is simply a marker for inflammation or is more directly involved in pathogenesis. In this paper, we attempt to resolve this issue by using the murine CIA model.

MATERIALS AND METHODS

Protocol for Induction of Murine CIA. DBA/1 male mice (6–8 weeks old) (Olac, Bichester, U.K.) were injected (intradermally) with 100 μl of native chicken type II collagen (Genzyme) (NC; 1 mg/ml in 0.1 M acetic acid) that had been emulsified with an equal volume of Freund's complete adjuvant (FCA) (Difco). Mice were housed five or six per cage. After 21 days, mice were challenged with a second intradermal injection of NC (FCA) and developed arthritis 8–14 days later. All injections were given under halothane anesthesia according to approved animal license protocols.

Parameters of CIA Model. *Arthritic index.* The arthritic index was determined by using the following scoring system for each joint: 0, no sign of inflammation; 1, difficulty in gripping cage; 2, obvious erythema; 3, obvious erythema with swelling. At the end of the experiment, joints were examined for histopathology. Joints were scored for erosions, polymorphonuclear infiltrates, pannus formation, and synovium infiltrates. The degree of joint pathology correlated well with the clinical assessments.

Type II collagen autoantibodies. For quantitation of type II collagen autoantibodies Nunc-Maxisorp 96-well plates were coated with 100 ng of murine type II collagen per well (generous gift of Tony Whyte) and then blocked with 1% bovine serum albumin in phosphate-buffered saline (PBS)

Abbreviations: CIA, collagen-induced arthritis; FCA, Freund's complete adjuvant; NC, native type II collagen; HDC, heat-denatured type II collagen.

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with 0.05% Tween 20. Sera were diluted 1:1000 in the PBS buffer and bound IgG antibodies were detected with a 1:1500 dilution of alkaline phosphatase-conjugated Fc-specific anti-mouse IgG (Sigma; A2429). After addition of *p*-nitrophenyl phosphate substrate for 20 min at 37°C, absorbance was measured at 405 nm. A standard curve of murine IgG using the same detecting antibody was used to convert the absorbance values to μg/ml.

Anti-type II collagen IgG secreting B cells. The number of cells was quantified using an enzyme-linked immunospot (ELISPOT)-type assay. Spleens were mechanically disrupted, and the cell suspension was passed through a 100-μm filter in balanced salt solution containing 5% heat-inactivated fetal calf serum. The lymphocytes were further purified by using a Histopaque discontinuous gradient (density, 1.09 g/ml) and spun for 20 min at 1000 × *g* at 18°C. Cells were suspended in RPMI 1640 medium with 2.5% heat-inactivated fetal calf serum, 2 mM glutamine, and 1 mM adenosine. Cells (10^6–10^7 in 500 μl) were transferred to Falcon Primaria plates (diameter, 3.5 cm) that had been precoated with bovine type II collagen (10 μg/ml) and incubated for 4 hr at 37°C in 5% CO_2/95% air. The cells were then removed using PBS with 0.05% Tween 20 and incubated with anti-mouse IgG alkaline phosphatase conjugate (Sigma; A5153) for 2 hr at 37°C. Spots were visualized with a solution containing 3 vol of 5 mM 5-bromo-4-chloro-3-indolyl phosphate in 10% diethanolamine (pH 9.8) and 1 vol of 3% low gelling agarose. As a positive control, total IgG antibody-secreting cells in the lymphocyte preparations were determined by coating the plates with protein A; plates coated with ovalbumin were used as negative controls in the ELISPOT assay. Approximately 14% of the total IgG-secreting cells in the spleen were found to secrete antibody that bound bovine type II collagen.

Agalactosyl IgG/GN7 reactivity. The relative concentration of G0 present in serum was determined by ELISA (12) using the anti-*N*-acetylglucosamine monoclonal antibody GN7 (a kind gift of G. A. W. Rook, University College, London). Briefly, ELISA plates were coated with 125 ng of protein A per well and blocked with 1% bovine serum albumin in PBS containing 0.05% Tween 20. Sera were diluted 1:500 in 100 mM glycine/150 mM NaCl, pH 8.0, and after a 2-hr incubation the plates were washed, and 100 μl of PBS was added. The plates were then floated on a water bath at 85°C for 15 min in order to denature the bound IgG and expose the interstitial carbohydrate residues. One microgram of biotinylated GN7 monoclonal antibody was then added per well. Visualization of the bound monoclonal antibody was achieved with a streptavidin–horseradish peroxidase conjugate and 2,2′-azinobis(3-ethylbenzthiazoline sulfonate) chromophore color development. Results are expressed as 650- to 490-nm absorbance values.

Time Course of Arthritis Induction Using Serial Bleeds from Individual Mice. Mice were injected with NC (FCA) as described in the protocol above for induction. At various time points 50 μl of blood was obtained by cardiac puncture after halothane anesthesia. The arthritic index, autoantibody, and percentage G0 levels were measured.

Production of IgG for Passive Transfer of Arthritis. Mice were injected with NC (FCA) as described in the induction protocol. At various time points after the first injection, groups of five mice were sacrificed, the spleen was taken for ELISPOT analysis, and the serum was collected and pooled. IgG was purified from the pooled sera using protein A. Aliquots (250 μg) of each IgG (see Fig. 2) were treated with 10 milliunits of β-galactosidase from *Streptococcus* strain 6646K (ICN/Flow Laboratories) in 50 μl of 50 mM citrate phosphate buffer (pH 6.5) containing 5 mM $MnCl_2$. Digests were performed at 37°C under a toluene atmosphere. After 18 hr, 100 μl of 100 mM Na_2HPO_4 was added and the IgG was repurified on protein A. Control digests were performed as described above but with β-galactosidase that had been placed in a boiling water bath for 15 min. The reaction was monitored by ELISA using the GN7 monoclonal antibody.

Induction of Arthritis by Passive Transfer. Recipient mice were primed by the induction protocol described, except that heat-denatured type II collagen (HDC) was used (10 min; 100°C). Twenty days after the second HDC/FCA injection, 125 μg of either β-galactosidase-treated or control IgG was injected in 100 μl of PBS via the tail vein. The arthritic index of each mouse was then regularly monitored.

Oligosaccharide Analysis. Protein A-purified IgG (250 μg) from pooled arthritic mouse sera was digested with either heat-denatured or active 6646K β-galactosidase as described. The samples were dialyzed and the N-linked oligosaccharides released by hydrazine were purified and radiolabeled by standard methods (13). The oligosaccharides were then digested with a mixture of neuraminidase, β-*N*-acetylglucosaminidase, and α-fucosidase. Precise conditions are given in ref. 14.

RESULTS

Relationship Between Autoantibody Levels, Agalactosyl IgG, and Arthritic Onset. Type II collagen autoantibody levels are elevated before arthritis onset; however, the increase in agalactosyl IgG determined by reaction with a monoclonal anti-*N*-acetylglucosamine antibody (GN7) appears more synchronous with arthritis onset. This was confirmed in serial bleed studies of several individual mice, one of which is shown in Fig. 1. Of seven mice, four showed large increases in GN7 reactivity around the arthritis onset date [average ΔGN7 = 0.34 (range, 0.10–0.80); average arthritis disease activity score = 7 (range, 5–9); disease onset days, 33, 39, 40, and 46]. Two mice developed a mild and transient late-onset arthritis (days 53 and 69) with no obvious increase in GN7 reactivity at

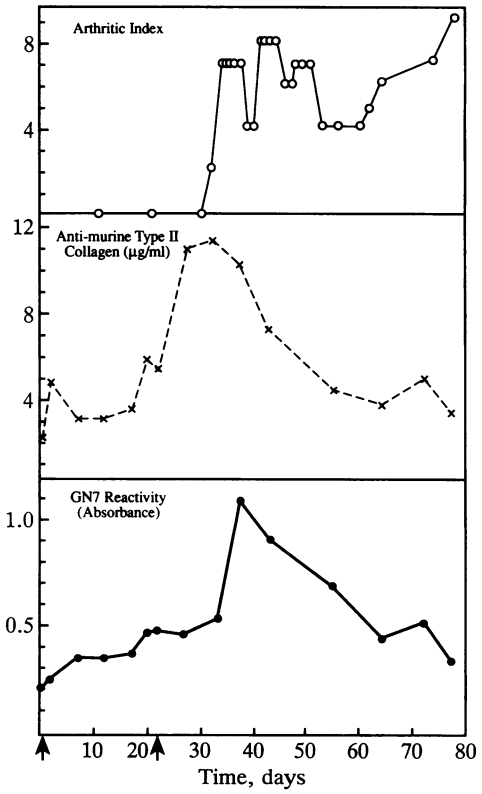

FIG. 1. Parameters of the CIA model were followed by serial bleeds from individual mice. The arthritic index (○) of individual mice was monitored as described. Serial bleeds from individual mice were analyzed for both IgG anti-type II collagen levels (x) and for GN7 reactivity (absorbance) (●). Representative data from a single mouse are shown. Arrows indicate NC (FCA) injection.

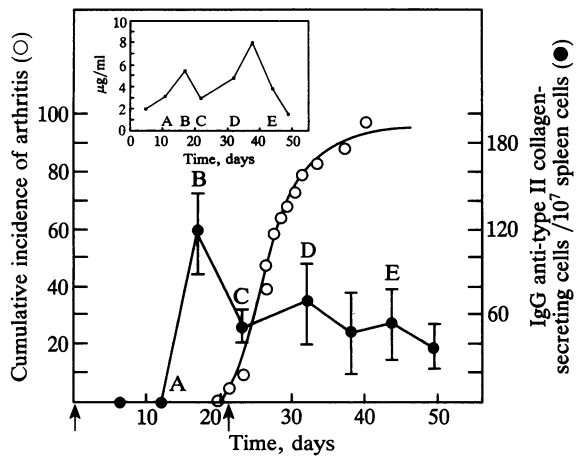

FIG. 2. Relationship between arthritis onset, splenic antibody-secreting cells, and serum autoantibody levels. Cumulative incidence of arthritis (○) was determined from the day of onset for each individual mouse. Anti-type II collagen IgG autoantibody levels are expressed as μg of pooled sera per ml (*Inset*); these were assayed essentially as described except 4 μg of purified IgG was added per well instead of dilute serum. Secreting cells measured by ELISPOT (●) are expressed as means ± SEM for four or five spleens. A total of eight time points were used; however, only those labeled A–E yielded sufficient IgG to be used in the later transfer study. Arrows indicate NC (FCA) injection.

disease onset and one mouse failed to become arthritic. It was observed that the serial cardiac bleeds delayed the onset date and the severity of the arthritis, presumably due to a stress-related corticosteroid effect. These results suggest that arthritogenic preparations of IgG may contain type II collagen autoantibodies of the agalactosyl IgG glycoform. To confirm this observation, passive transfer of arthritis with different IgG glycoforms was performed from groups of mice sacrificed at different time intervals.

Source of IgG for Passive Transfer and Effect of β-Galactosidase Treatment. Four or five mice were sacrificed at each of the eight time points. IgG was purified from the pooled sera at the time points shown in Fig. 2. However, only five pools provided sufficient material to be used in subsequent transfer experiments (A–E). This figure also shows that large numbers of anti-type II collagen IgG-secreting cells are present in the mice spleens around day 17 of arthritis induction. Fig. 2 (*Inset*) also confirms that peak type II collagen autoantibody

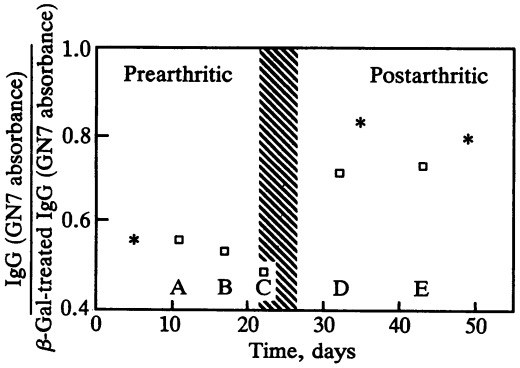

FIG. 3. Assessment of the agalactosyl content of pooled IgG in the transfer experiment using the GN7 monoclonal antibody assay. Data are expressed as the ratio of GN7 absorbance of the control IgG digest to the corresponding GN7 absorbance of the β-galactosidase-treated IgG. Shaded area represents the time for 50% of the mice to become arthritic. *, Samples were not used in the transfer study, as insufficient material was available. For fully galactosylated IgG (G0 = 0%), the value would be 0; for fully agalactosylated IgG, the value is 1.

levels occur around days 17 and 38 with the first peak preceding the mean onset date of arthritis by 10 days.

The pooled IgG fractions were treated with β-galactosidase as described and analyzed by GN7. Fig. 3 shows that the β-galactosidase not only caused an increase in the GN7 reactivity/agalactosyl content of the test samples (up to 100% as drawn in the figure) but that sometime between days 22 and 32 there was a marked shift in the basal GN7 reactivity of the IgG. This implies that the prearthritic IgG is more fully galactosylated and therefore has even greater capacity for change when treated with 6646K β-galactosidase compared to postarthritic IgG. We confirmed that this enzyme is active on native murine IgG by formal oligosaccharide exoglycosidase digestion and sequencing (data not shown).

Dose-Dependent Passive Transfer of Arthritis with IgG and Galactose-Depleted IgG. IgG was obtained from a group (n = 10) of arthritic mice and injected via tail vein into a group (n = 3) of recipient mice that had been primed with HDC (FCA) as shown in Fig. 4*a*. A two-injection protocol was used as described by Seki (15). All mice in the group developed acute synovitis followed by persistent arthritis. These results are in complete agreement with the data of Seki *et al.* (15), including the severity of arthritis. The histopathology of the joint changes has been described (15). A series of pilot experiments demonstrated that the minimum single dose with which we could just observe an arthritic response was ≈125 μg.

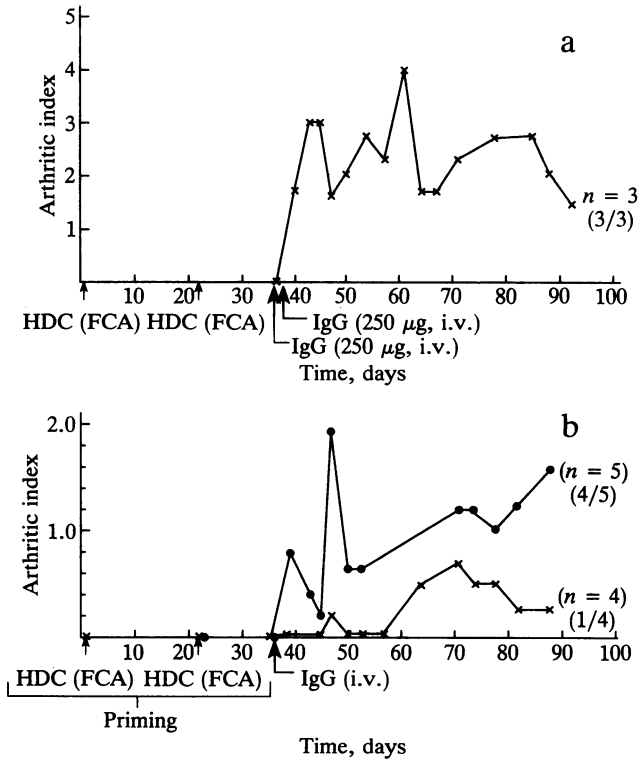

FIG. 4. (*a*) Induction of chronic persistent arthritis by passive transfer of IgG. Purified IgG from mice with arthritis is able in passive transfer experiments to cause both acute and chronic arthritis. The standard protocol is two injections (250 μg × 2) via tail vein of pooled IgG after priming HDC (FCA). (*b*) Enhancement of the pathogenic effect of IgG by β-galactosidase. IgG used for passive transfer was obtained from the five pools (A–E) of IgG shown in Fig. 2. Each pool of IgG was treated with β-galactosidase and repurified before injection. Incidences are given for acute synovitis. Results are in agreement with the expected increase in IgG0, which would take place after β-galactosidase treatment. β-Galactosidase increases the percentage G0 from 32% to 72%, a 2.3-fold increase. ●, Arthrogenic IgG (110 μg) (β-galactosidase digested); x, arthrogenic IgG (110 μg) (heat-denatured β-galactosidase).

Passive Transfer of Arthritis with IgG and Galactose-Depleted IgG from Donor Mice at Different Stages in Development of Disease. To determine whether the amount of agalactosyl IgG in the arthritogenic preparations was critical for passive transfer, a group of 10 mice identically matched for age, sex, and housing were used as recipients. The IgG used for passive transfer was obtained from the five pools (A–E) of IgG indicated in Fig. 2. To determine whether the amount of agalactosyl IgG in the preparation was limiting, we treated each pool of IgG with β-galactosidase. Fig. 4*b* shows that the mice receiving IgG (125 μg) treated with the β-galactosidase from four of the five pools developed both acute synovitis (days 0–14) and chronic persistent arthritis. In contrast, only one pool of IgG (125 μg) not treated with β-galactosidase was able to induce a very slight acute response. These data suggest that in addition to anti-type II collagen antibodies, the agalactosyl IgG content is an additional important factor in the passive transfer of arthritis.

Evidence that the anti-type II collagen antibodies themselves need to be of the agalactosyl IgG glycoform can be obtained from an analysis of which pools of IgG shown in Fig. 2 were able to support passive transfer. Fig. 5 shows an individual breakdown analysis of the pool data shown in Fig. 4*b*. IgG from pools A–E could not induce acute synovitis with the exception of recipients of pool D IgG, who developed a very mild synovitis around day 50. It is of interest to note, however, that these same mice developed a mild chronic arthritis 30–50 days after the infusion, with the exception of recipients of group A IgG, as shown in Fig. 4*b*. Group A IgG is from animals sacrificed before the development of anti-type II collagen antibodies and group D IgG is from arthritic animals near the day of peak titer as shown in Fig. 2. IgGs from pools B–E when treated with β-galactosidase were able to induce an acute but transient synovitis that remitted with the characteristic clearance rate of IgG. At around 5 days postinfusion (day 80), a chronic arthritis became evident in all recipient mice except for those receiving IgG from pool A. These data strongly suggest that the presence of neither agalactosyl IgG alone nor anti-type II collagen autoantibodies alone is sufficient to support passive transfer; anti-type II collagen antibody of the agalactosyl IgG glycoform is required.

That the chronic arthritis was related to the IgG infusions and not simply age associated can be inferred from the observation that mice injected with IgG containing no anti-type II collagen (pool A) did not develop late-onset arthritis. The lack of response to the IgG infusion in some of these mice may have been because they were nonresponders. Therefore, at the end of the experiment all mice were challenged with a single NC (FCA) intradermal injection (data not shown) and subsequently developed arthritis.

DISCUSSION

Serial bleed studies of individual mice confirmed that there is a greatly increased expression of agalactosyl IgG glycoforms present in IgG obtained at the time of arthritis onset as measured by an increase in GN7 reactivity of the serum IgG (Fig. 1). Similar observations were made for pooled IgG from groups of mice sacrificed at different time intervals after priming and challenge with type II collagen in FCA (Fig. 3). These results complement our previous study in which we monitored agalactosyl IgG isolated from serial bleeds of mice in pregnancy-induced remission of type II CIA (9). We carried out independent experiments following either seven individual mice with serial bleeds or groups of five mice all sacrificed at a single time point. Comparison of the data from the two groups indicated that the serial bleeds interfered slightly with the time course and response of the animal. This is why data are shown for both serial bleeds and groups.

Of interest is our observation reported here and by others (15) that the overall serum anti-type II collagen autoantibody

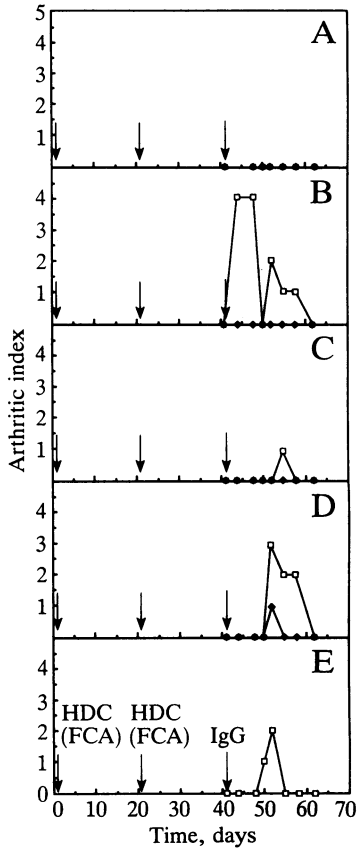

FIG. 5. Individual analysis of pool data. Effects of the transfer of pooled IgG purified from 11-day (*A*), 17-day (*B*), 22-day (*C*), 32-day (*D*), and 44-day (*E*) collagen II (FCA) immunized mice are shown. □, With β-galactoside; ◆, without β-galactoside.

levels in mice with chronic CIA is not raised: indeed, we find that the decline in serum concentration begins at the day of arthritis onset (Fig. 1 *Top* and *Middle*) despite the continued presence of antibody-secreting cells in the spleen (Fig. 2). The results suggest that the drop in titer arises either from the rapid removal of the agalactosyl autoantibodies by *in situ* deposition on cartilage or from fluid phase immune-complex formation followed by clearance. This is also consistent with our observation that DBA/1 mice immunized with type II collagen in Freund's incomplete adjuvant (i.e., no mycobacteria) develop a higher level of autoantibodies to type II collagen than mice immunized with collagen in FCA but do not develop arthritis. No elevation in agalactosyl IgG is found in these mice (data not shown). This observation, taken with the role of agalactosyl IgG glycoforms of autoantibody in initiating synovitis as demonstrated by the passive transfer experiments, suggests that one important role of the T cell in chronic disease may be to maintain a high agalactosyl IgG glycoform specific activity rather than a high titer of autoantibody.

Three types of passive transfer experiments have previously been performed. These are (*i*) passive transfer of serum concentrates containing antibodies to type II collagen obtained from arthritic mice (15–17), (*ii*) passive transfer of purified type II collagen antibody (11, 18, 19), and (*iii*) passive transfer of monoclonal antibodies with specificities directed toward type II collagen (20, 21).

The results of the passive transfer studies reported here are consistent with the results of Seki *et al.* (15), who demonstrated that passive transfer of IgG purified from mice with CIA is able to elicit an acute synovitis and chronic arthritis in recipients primed with heat-denatured collagen. Infusion of IgG from arthritic mice or monoclonal antibody mixtures to collagen into naive recipient mice results in only a transient

arthritis, which remits over a 20-day period as the passively transferred IgG is cleared from the system (15, 16, 18). Mice primed with HDC develop reactive T cells identical to those present in mice primed with native collagen, but these mice do not develop arthritis (15). Priming and challenge with heat-denatured collagen do not elicit a B-cell response since a native triple-helical conformation is required for recognition of the relevant epitope(s) by resting collagen-specific autoreactive B-cell clones (1).

Some, but not all, passive studies with purified type II collagen antibody have been able to demonstrate a direct link between the induction of humoral autoimmunity to type II collagen and the initiation of arthritis (11, 15–19, 22). Histopathology studies have shown that the synovitis associated with passive transfer of high doses of polyclonal type II collagen antibody is initiated by the activation of complement on the cartilage surface, followed by synovial infiltration of neutrophils and other mononucleated cell types (19, 23). However, passive transfer studies with single monoclonal antibodies have been unsuccessful in inducing arthritis (20). This has led Holmdahl *et al.* (22) to speculate that a collection of components in the polyclonal arthritogenic IgG preparations (e.g., rheumatoid factors, anti-collagen, anti-idiotype antibodies, anti-C1q, etc.) may be of importance. Terato *et al.* (21) have recently shown that if at least three different anti-type II collagen monoclonal antibodies of differing specificity are infused simultaneously, arthritis develops. This observation led to the hypothesis that some spatial or quantitative relationship of antibody was necessary on the cartilage surface before complement could be activated.

Importantly, we have now been able to demonstrate (Fig. 4*b*) that if the IgG preparation used for passive transfer is first digested with β-galactosidase to increase the level of the agalactosyl IgG glycoform level, then IgG is more effective in the passive transfer. This indicates that the glycosylation status of the autoantibody is at least one factor in determining whether an autoantibody is pathogenic. By implication, we have assumed that the agalactosyl IgG anti-type II collagen is the pathogenic form, but we stress that we have not formally proven this. Such experiments will require the purification of this antibody in an amount sufficient for determination of the associated oligosaccharide structure. However, the concentration of autoantibodies is very small indeed (μg/ml), so this represents a considerable experimental challenge for the future.

It is a common observation that many nonsusceptible strains of mice elicit anti-collagen titers higher than arthritis-susceptible strains. Similarly, the autoantibody response within susceptible strains is not always indicative of the development of the disease (24). For example, anti-type II collagen IgG isolated from the H-2q SWR strain is arthrogenic when transferred to DBA/1 mice, while no arthritis is obvious in the animals from which it is obtained (25). In general, collagen epitopes reactive with antibodies are cryptic in *in vitro* assays and made accessible only after protease digestion of normal joint tissue (26) or after infusion of arthrogenic anti-type II collagen *in vivo* (27). Such a protease may also be important in mediating the action of the agalactosyl IgG glycoforms. The x-ray data on IgG have demonstrated that the oligosaccharide residues in IgG are inaccessible (5). For example, reactivity with the anti-*N*-acetylglucosamine monoclonal antibody GN7 is only possible after heat denaturation of the IgG (12). Reactivity of IgG with the β-galactosidase from group A *Streptococcus* strain 6646K used in this study appears to be an exception. Indeed, the role of β-galactosidase as a bacterial or viral virulence factor may need further examination.

The pathology associated with antibody-induced arthritis (16) has the mixed characteristics of an antibody-mediated hypersensitivity reaction (28). Clearly the data presented here demonstrate that the agalactosyl IgG glycoform plays a major role in pathogenesis but any link between agalactosyl IgG and initiation of the inflammation remains to be established. Of relevance are our recent demonstrations that repeated intraperitoneal injections of interleukin 6 (20 μg twice a day for 5 days) result in a sustained increase in agalactosyl IgG expression in DBA/1 mice (unpublished data). Interleukin 6 dysregulation is thought to contribute to the pathogenesis of rheumatoid arthritis (29) and elevated levels of interleukin 6 are present in mice with CIA (30).

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