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Kinetic Analysis of Interaction of Different Types of Rheumatoid Factors with Immobilized IgG Using Surface Plasmon Resonance[1]

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Rheumatoid factors (RFs) are autoantibodies, which recognize antigens on a constant region of immunoglobulin G (IgG). Among various RF classes, RF of the IgG class (IgGRF) forms immune complexes in rheumatoid joints and is implicated in the pathogenesis of rheumatoid arthritis (RA). To characterize the formation of IgGRF immune complexes, in the present study, IgGRF was isolated from sera of RA patients, and its interaction with immobilized IgG was analyzed and compared to that of IgMRF or IgARF by means of surface plasmon resonance. On gel filtration, the IgGRF was eluted as a single peak corresponding to IgG, excluding the possible formation of self-associating IgGRF complexes in solution. Sensorgrams of the interaction of IgGRF with immobilized IgG revealed that it clearly bound to the IgG at 6°C, but not at 30°C. The degree of interaction decreased inversely with an increase in temperature, suggesting that IgGRF is much more reactive at lower temperatures. In contrast, the interaction of IgARF and IgMRF with IgG at 6°C was similar to that at 30°C. The association rate constant (k_a) of IgGRF decreased with an increase in temperature, while those of IgARF and IgMRF were similar under various thermal conditions. The dissociation rate constant (k_d) of IgGRF was greatly reduced at 25°C, but those of IgARF and IgMRF slightly increased with an increase in temperature. These results suggested that the mode of interaction of IgGRF with IgG differed from in the cases of IgMRF and IgARF. The kinetic properties of the IgGRF-IgG interaction may facilitate elucidation of the IgGRF immune complex formation in rheumatoid joints.

Key words: immunoglobulin G, rheumatoid arthritis, rheumatoid factor, surface plasmon resonance.

Rheumatoid factors (RFs) are autoantibodies reactive with the constant region of IgG (*1*). They are found at high concentrations in the blood and inflammatory tissues of patients with rheumatoid arthritis (RA) (*2*). It has been shown that rheumatoid contains abundant aggregates of IgG and RF (*2*), and therefore RF may contribute to immune complex formation, complement consumption, and chronic tissue damage in the rheumatoid synovium (*3*). RF reactivity has been detected in all immunoglobulin isotypes, and IgMRF has been the most frequently studied (*1*, *4*). However, IgGRF is considered to be more important in the pathology of RA. IgGRF is the most frequently synthesized antibody in the rheumatoid synovium (*5*), and these immunoglobulins associate with each other to form large immune complexes in the synovial fluid (*2*, *6*). Despite the close association of IgGRF immune complexes with the pathology of RA, the mechanism of formation of these complexes remains unclear. It has been shown that the Fc binding activity of IgG4 is localized in the constant region of IgG, suggesting that IgGRF may not be an actual antibody (*7*). Since the galactose-lacking form of IgG is predominantly present in pathological IgG aggregates in rheumatoid joints (*8*, *9*), it has been postulated that this galactose-lacking form self-aggregates through a mechanism different from the classical antigen-antibody interaction (*10*). On the other hand, sequence analysis of the VH region of IgGRF has indicated a somatic mutation characteristic of an antigen-driven response (*11*). To understand the relationship between IgGRF immune complexes and RA pathology, biochemical characterization of the formation of IgGRF immune complexes is essential.

In the present study, we applied surface plasmon resonance detection to analysis of the IgGRF-IgG interaction. This recently developed method allows real time detection of molecular interactions (*12*, *13*) and permits analysis of several kinetic parameters. With this detection method, we analyzed the kinetic parameters of the interaction between IgGRF and immobilized IgG, and compared them to those in the cases of other classes of RFs to elucidate the formation of IgGRF immune complexes. Our results suggested that IgGRF was more reactive with immobilized IgG at

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Abbreviations: IgARF, IgA with RF activity; IgGRF, IgG with RF activity; IgMRF, IgM with RF activity; mAb, monoclonal antibody; RA, rheumatoid arthritis; RF, rheumatoid factor.

lower temperatures, and that the interaction between IgGRF and IgG differed kinetically from those in the cases of other classes of RFs.

MATERIALS AND METHODS

Equipment and Materials—The BIAcore X system, sensor chip CM5, and amine coupling kit used were obtained from Biacore AB (Uppsala, Sweden). Human IgG, IgM, and IgA were purchased from Sigma-Aldrich (Tokyo). IgG-Sepharose 6 Fast Flow was from Amersham-Pharmacia Biotech (Tokyo). Anti-human Fc region monoclonal antibodies of the mouse IgG1 subclass (clone SB7H6) and mouse IgMκ subclass (clone G-01) were from Biogenesis Inc. (Sandown, NH) and Yamasa (Tokyo), respectively. Anti–human IgG, anti–human IgM, anti–human IgA, anti–human IgD, and anti-human IgE antibodies were from Medical & Biological Laboratories (MBL, Nagoya). The Silver Stain II kit used was from Wako Pure Chemicals (Osaka).

Preparation of RFs from Sera of RA Patients—All procedures for the separation of RFs were carried out at 4°C. Pooled sera were obtained from 10 RA patients (females with an average age of 47.2 ± 3.6 years) and dialyzed against 15 mM phosphate buffer, pH 8.0, followed by chromatography to isolate the IgG fraction on a column of DEAE-cellulose (DE-52 from Whatman International Maidstone, UK). The pass-through fraction [DE(–)] was used as the purified IgG after being subjected to chromatography on a Superdex 200 HR (10/30) column (Amersham-Pharmacia Biotech.), and the fraction which adsorbed to the DEAE-cellulose column [DE(+)] was used as the non-IgG fraction. The total IgG was eluted from the Superdex 200 HR column as a single peak corresponding to the elution position of human IgG. Three IgGRF fractions were isolated from the purified IgG obtained from the pooled sera by means of chromatography on an IgG-Sepharose column (1.5 × 20 cm). In brief, the purified IgG was dialyzed against 50 mM Tris-HCl, pH 7.6, containing 150 mM NaCl and 0.05% Tween 20 (TBS-T), and then applied and recirculated at 4°C for 12 h through an IgG-Sepharose column equilibrated with the TBS-T at the flow rate of 1 ml/min. The column was washed with TBS-T until the absorbance of the eluate at 280 nm was zero, and then eluted with 0.1 M Glycine/HCl, pH 3.0, to obtain RF. The pass-through fractions were collected and used for further chromatography. The RF samples which were eluted from the IgG-Sepharose column were immediately neutralized with 1 M Tris-HCl (pH 8.0) to pH 7.0 and used as the IgGRF fraction [DE(–)IgG(+)]. The pass-through fraction from the first IgG-Sepharose column [DE(–)IgG(–)] was then rechromatographed on the same column. The adsorbed fraction was used as the second IgGRF fraction [DE(–)IgG(–)(+)]. The pass-through fraction from the second column [DE(–)IgG(–)(–)] was again rechromatographed on the same column, and the adsorbed fraction was used as the third IgGRF fraction [DE(–)IgG(–)(–)(+)]. The pass-through fraction from the third column was used as the non-RF IgG [DE(–)IgG(–)(–)(–)]. Each IgG sample was then subjected to chromatography on a Superdex 200 HR (10/30) column equilibrated with HBS (10 mM Hepes, pH 7.4, with 0.15 M NaCl and 0.005% Tween 20) at 4 and 25°C. All IgG samples were each eluted from the column as a single peak at the same position as that of human IgG.

IgMRF and IgARF were purified from the non-IgG fraction of sera of RA patients [DE(+)] by IgG-Sepharose column chromatography, followed by gel filtration on a Superose 6 HR (10/30) column (Amersham-Pharmacia Biotech) equilibrated with HBS. IgM (970 kDa), IgA dimer (390 kDa), and IgA monomer (160 kDa) were eluted separately from the Superose 6 HR column (see Fig. 1C). Since the IgA monomer was a major component of the IgA in the pooled sera of the patients, the IgA monomer and IgM fractions were collected and used as IgARF and IgMRF, respectively. The protein concentration of each sample was determined by means of a standard protein assay (Protein Assay Reagent from Bio-Rad Laboratories, CA) based on Bradford's method using human IgG (Sigma-Aldrich Japan, Tokyo) as the standard protein. The protein concentrations of the IgGRF [DE(–)IgG(+)], IgMRF, and IgARF fractions were 145, 27.0, and 94.0 μg/ml, respectively. The purities of IgGRF, IgMRF, and IgARF were confirmed by SDS-PAGE using Laemmli's system (*14*), followed by Western blotting using monoclonal antibodies against human IgG, IgM, IgA, IgD, and IgE, respectively.

Measurement of RF Reactivity of the Purified RFs—The specific RF reactivity of each IgGRF fraction was determined as its ability to bind to the immobilized IgG Fc regions on ELISA, using an anti–human IgG-Fd region antibody-conjugated enzyme (Eitest IgGRF test kit; Eisai, Tokyo). Absorbance was measured at a wavelength of 405 nm and a reference wavelength of 490 nm with a Model 450 Microplate Reader (Bio-Rad Laboratories, CA). The IgGRF index was calculated from the absorbance data according to the following formula:

$$\text{IgGRF index} = F\text{–}B/FN\text{–}BN,$$

where F is the absorbance of the sample on an Fc-coated well, B the absorbance of the sample on a BSA-coated well, FN the absorbance of normal IgG on an Fc-coated well, and BN the absorbance of normal IgG on a BSA-coated well. The RF reactivities of the IgMRF and IgARF fractions were basically determined with the Eitest IgGRF test kit as for determination of the RF reactivities of the IgGRF fractions, but an anti–human IgM antibody and an anti–human IgA antibody conjugated with horseradish peroxidase were used as the secondary antibodies instead of the anti–human IgG-Fd region antibody provided with the kit. Binding of the secondary antibodies was visualized with 0.1% H_2O_2 and 2,2′-azino-di-[3-ethylbenzthianozoline-6-sulfonic acid] at an absorbance level of 415 nm. The RF reactivities of IgMRF and IgARF were estimated from the difference in absorbance (ΔA_{415}) between Fc-coated wells and BSA-coated wells. The specific RF reactivity was taken as the IgGRF index and ΔA_{415} in 1 mg of protein of each sample.

Immobilization of Human IgG on a Sensor Chip—Immobilization of human IgG and BSA on the surface of a sensor chip CM5 was performed as described in a previous report (*15*). A continuous flow of HBS (10 mM Hepes, pH 7.4, with 0.15 M NaCl and 0.005% Tween 20) was maintained over the sensor surface. The carboxyl groups on the surface were activated by injecting a solution of 0.2 M *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide and 0.05 M *N*-hydroxysuccinimide. Specific surfaces were obtained by injecting intact human IgG over the activated surface of flow cell 1 (Fc1) of the chip. To obtain a control surface, BSA was injected into flow cell 2 (Fc2) of the same chip. The immobi-

lization procedure was completed by injecting 1 M ethanolamine hydrochloride to block the remaining ester groups. About 15,000 RU of intact human IgG and 12,000 RU of BSA were immobilized on Fc1 and Fc2 of a sensor chip CM5, respectively, which was then used as the sensor chip containing the immobilized intact IgG. RU represents the amount of protein immobilized on the chip. One thousand RU is equivalent to 1 ng/ mm^2.

Interaction Assay—All assays were performed with BIAcore X equipment using the sensor chip with the immobilized intact IgG. The proteins were dialyzed against HBS and diluted in the same buffer to an appropriate concentration. A continuous flow of HBS (5 μl/min) was maintained over the sensor surface, and the proteins diluted in HBS were then injected for 7 min over the surfaces of the chips at the flow rate of 5 μl/min. After 7 min, a continuous flow of HBS (5 μl/min) was maintained over the sensor surface again. For the standard assay, 5.0, 3.125, and 3.125 μg/ml of the IgGRF, IgMRF, and IgARF samples were used, respectively, and 3.125 μg/ml of an anti-human Fc mouse monoclonal IgG antibody or an anti-human Fc monoclonal IgM antibody was used for testing the samples. In each case, appropriate concentrations of proteins were employed. Specific sensorgram data were obtained by subtraction of the sensorgram results obtained on Fc2 (BSA surface) from those obtained on Fc1 (specific human IgG surface). After completing the analysis, the surface of the chip was regenerated by injecting 35 μl of regeneration buffer (0.1 M Glycine-HCl, pH 2.98, containing 1 M NaCl), followed by washing with HBS, and then the next sample was injected.

Kinetic Analysis—The association and dissociation rate constants (k_a and k_d) were determined from at least two sensorgrams with different concentrations of proteins using BIA evaluation software (Ver. 3.0) linked to global analysis. Linear curve fitting was applied to the entire data set for global analysis. The association rate (k_a) and dissociation rate (k_d) constants were calculated from the sensorgram values between 10 and 403 s (association phase), and those between 542 and 823 (dissociation phase). Based on the k_a and k_d values, the dissociation constants ($K_D = k_d/k_a$) were estimated. For calculation of kinetic parameters for the interaction of immunoglobulins with immobilized human IgG, the following molecular weights were used; 150,000 for IgG, 160,000 for IgA monomer, and 970,000 for IgM. The resonance response of each sample was determined from the average resonance units between 393 and 402 s of the sensorgrams.

RESULTS

Purification of RFs of Different Classes of Immunoglobulin—We purified total IgG from the pooled sera of 10 RA patients (females with an average age of 47.2 ± 3.6 years) by DEAE-cellulose column chromatography. The total purified IgG from these patients migrated as a single band on SDS-PAGE under non-reducing conditions (Fig. 1A, lane a), and dissociated into heavy and light chains under reducing conditions (Fig. 1A, lane b). Western-blotting revealed that the total IgG fraction [DE(–)] only contained IgG, *i.e.* it was not contaminated by other immunoglobulins or serum proteins (Fig. 1A, lanes c, d, and e). Three IgGRF fractions, [DE(–)IgG(+), DE(–)IgG(–)(+), and DE(–)IgG(–)(–)(+)], were then separated from the highly purified total IgG fraction based on their ability to bind to an IgG-Sepharose column. To determine whether or not the isolated IgGRF samples could form self-aggregates in solution, each sample was subjected to chromatography on a Superdex 200 HR column equilibrated with HBS. Each IgGRF sample was eluted from the column at 4 or 25°C as a single peak (about 150 kDa) corresponding to monomeric IgG (Fig. 1B), excluding the possible formation of self-associating IgGRF complexes in the solution. We also purified IgMRF and IgARF fractions from the DEAE-cellulose–adsorbed fraction [DE(+)] of the same pooled sera by chromatography on the same IgG-Sepharose column followed by chromatography on a Superose 6 HR column equilibrated with HBS (Fig. 1C). The peaks of the proteins which were eluted at the same positions as human IgM and human IgA monomers were collected and used as the IgMRF and IgARF samples, respectively (see below). Silver staining and Western blotting with anti-IgM, anti-IgA, and anti-IgG antibodies revealed that the IgMRF and IgARF samples only contained the IgM and IgA subclasses, respectively (Fig. 1D), *i.e.* they were not contaminated by IgG. This strongly indicated that IgMRF and IgARF did not form immune complexes with IgG. In addition, IgD and IgE could not be detected in the IgMRF and IgARF samples on Western blotting with an anti–human IgD antibody and an anti–human IgE antibody, respectively (data not shown).

Determination of RF Reactivities Using Surface Plasmon Resonance—In order to determine whether or not the isolated IgGRF fractions which bound to the IgG-Sepharose column had RF reactivity, the RF reactivity of each fraction was determined by means of ELISA using the Fc region of human IgG as an immobilized antigen. All isolated IgGRF fractions which bound to IgG-Sepharose interacted much more strongly with the immobilized Fc region of human IgG than the total IgG fraction did (Fig. 2A). In addition, the binding of the IgMRF and IgARF fractions to the immobilized Fc region of human IgG was significantly higher than that of the normal human IgM and human IgA, respectively (Fig. 2B). Therefore, the IgGRF, IgMRF, and IgARF fractions were considered to have RF reactivity. Interestingly, the specific RF reactivity of each of the three IgGRF fractions increased in the order of DE(–)IgG(–)(–)(+) < DE(–)IgG(–)(+) < DE(–)IgG(+) (Fig. 2 A). These results indicated that each of the IgGRFs which bound differently to the IgG-Sepharose column had a different specific RF reactivity. We then analyzed the interaction between IgGRF and human IgG immobilized on a sensor chip by means of surface plasmon resonance (Fig. 2C). When the same concentration (5 μg/ml) of each IgGRF fraction was injected, IgGRF, which had exhibited the highest specific RF reactivity [DE(–)IgG(+)] on ELISA, exhibited the highest resonance response to the immobilized human IgG. The resonance units obtained with the three IgGRF fractions increased relative to the specific RF reactivity, which had been determined by ELISA, *i.e.*, DE(–)IgG(–)(–)(+) < DE(–)IgG(–)(+) < DE(–)IgG(+). Interaction between the non-RF IgG [DE(–)IgG(–)(–)(–)] and immobilized human IgG could not be detected with this method. Since the results obtained using surface plasmon resonance were consistent with those of ELISA, this method of detection can be used for analysis of the formation of autoimmune complexes involving IgGRF. In the following experiments, we used the DE(–)IgG(+) fraction as the IgGRF sample in order to in-

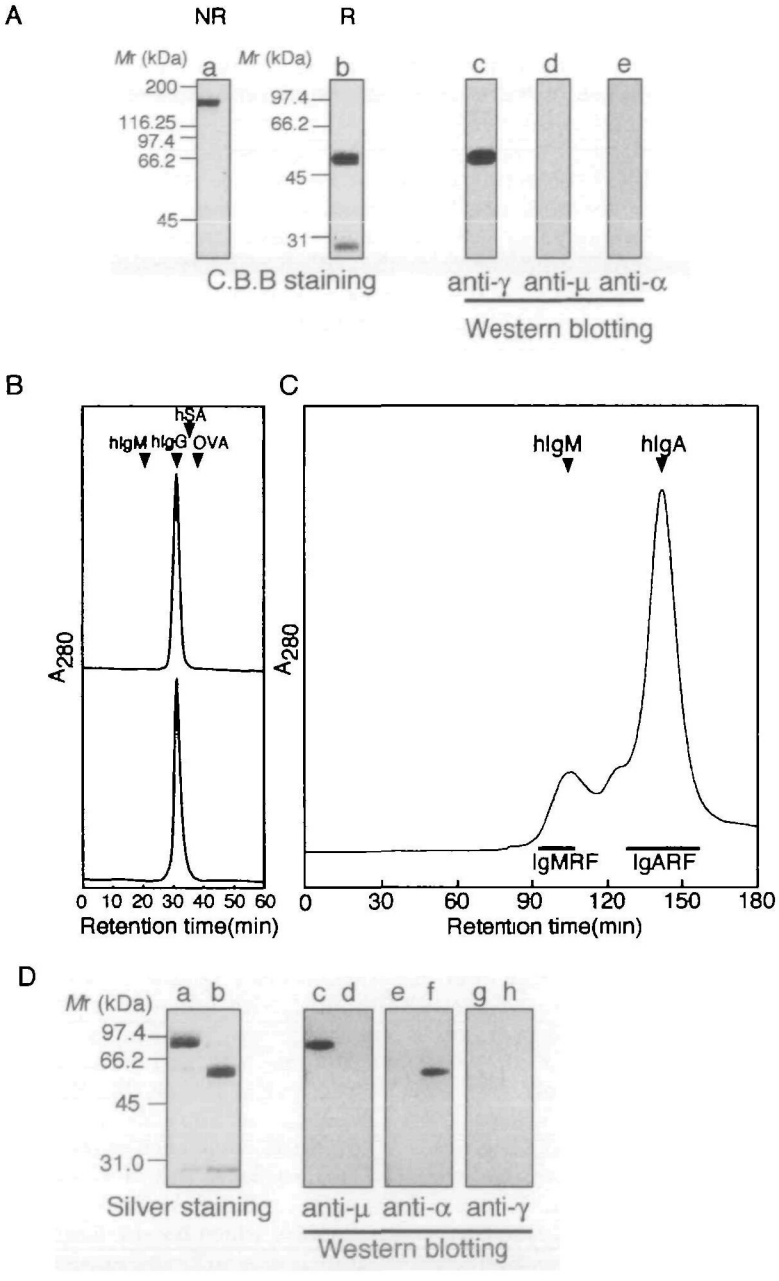
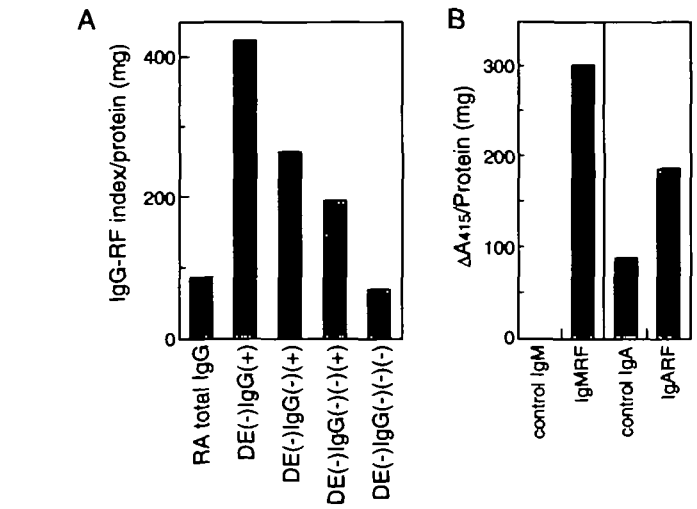


Fig. 1. **Analysis of the IgG, IgM, and IgA fractions bound to the IgG-Sepharose column.** A: The total IgG [DE(–)] fraction purified from pooled sera of patients with rheumatoid arthritis was subjected to SDS-PAGE on a 5–20% gradient gel under non-reducing conditions (NR, lane a) and on a 10% gel under reducing conditions (R, lane b). The gels were then stained with Coomassie Brilliant Blue R-250 (C.B.B.). The total IgG fraction was subjected to SDS-PAGE on a 10% gel under reducing conditions, followed by Western blotting. The membranes were then stained with an anti–human IgG (γ-chain) rabbit antibody (lane c), an anti–human IgM (μ-chain) rabbit antibody (lane d), or an anti–human IgA (α-chain) rabbit antibody (lane e). The molecular weights of protein markers are indicated on the left of the panels. B: The IgGRF [DE(–)IgG(+)] fraction was subjected to chromatography on a Superdex 200 HR (10/30) column equilibrated with HBS at 4°C (top) and 25°C (bottom). Arrowheads indicate the elution positions of human IgM (hIgM), human IgG (hIgG), human serum albumin (hSA), and ovalbumin (OVA), respectively. C: The non-IgG fraction of RA patient sera [DE(+) fraction] was subjected to chromatography on an IgG-Sepharose column, and the resulting IgG-Sepharose–adsorbed fraction [DE(+)IgG(+)] was subjected to chromatography on a Superose 6 HR (10/30) column equilibrated with HBS. Arrowheads indicate the elution positions of authentic human IgM (hIgM) and human IgA monomer (hIgA), respectively. The fractions indicated by bars were collected and used as IgMRF and IgARF, respectively, after being rechromatographed on the same column. D: The IgMRF (lanes a, c, e, and g) and IgARF (lanes b, d, f, and h) fractions were subjected to SDS-PAGE on a 10% gel under reducing condition, followed by Western blotting. Lanes a and b were stained with silver; lanes c and d, with an anti–human IgM (μ-chain) rabbit antibody; lanes e and f, with an anti–human IgA (α-chain) rabbit antibody; and lanes g and h, with an anti–human IgG (γ-chain) rabbit antibody. The molecular weights of protein markers are indicated on the left of the panels.

vestigate the mode of interaction between IgGRF and IgG since this fraction exhibited the highest resonance response.

Determination of the Mode of Interaction between RFs and Intact IgG—The interactions between different immunoglobulin subclasses with RF reactivity and human IgG were compared using surface plasmon resonance. As standard immunoglobulins that interact with the Fc region of human IgG, an anti-human Fc mouse monoclonal IgG antibody (anti–Fc IgG mAb) and an anti–human Fc mouse monoclonal IgM antibody (anti–Fc IgM mAb) were used. Sensorgrams of the interactions of different classes of immunoglobulins exhibiting RF reactivity and the anti-Fc mAb with the sensor chip–immobilized human IgG are shown in Fig. 3. The anti–Fc IgG mAb and anti–Fc IgM mAb associated with the immobilized intact human IgG much more strongly at 25°C than at 6°C (Fig. 3, D and E). The interaction of IgMRF with the IgG was almost the same at both temperatures (Fig. 3B). On the other hand, the interaction of IgGRF with the immobilized IgG was clearly observed at 6°C, but was very weak at 25°C (Fig. 3A). It is possible that the weak interaction of IgGRF observed with surface plasmon resonance was due to a decrease in the monomeric form of IgGRF molecules because of the formation of IgGRF-IgGRF complexes in the sample at 25°C. However, the IgGRF fraction was eluted as a single peak from the Superdex 200 HR column at a position corresponding to that of authentic human IgG even at 25°C (Fig. 1B), suggesting that most of the IgGRF molecules in the solution at this temperature were in the monomeric form. Therefore, the interaction of the IgGRF molecules with the immobilized human IgG was obviously weaker at 25°C



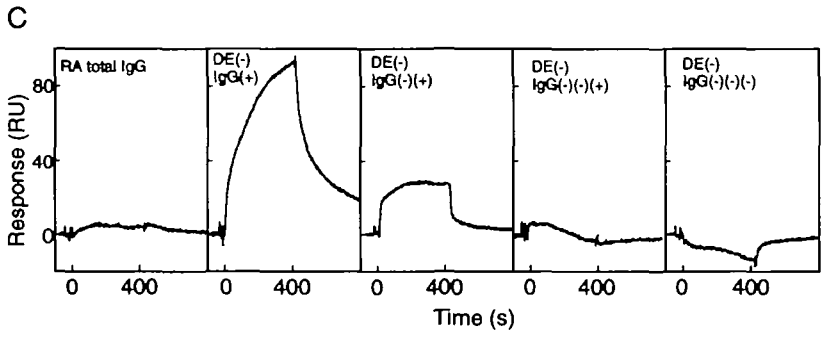


Fig. 2. **Specific RF reactivities of the IgGRF, IgMRF, and IgARF fractions.** A: RF reactivities of the IgGRF fractions. The RF reactivities of the IgGRF fractions were determined by measuring the binding of IgG to the Fc region on IgG-coated plates, as described under "MATERIALS AND METHODS." The specific IgGRF index was calculated as the amount of IgGRF per 1 mg protein in each sample. B: RF reactivities of the IgMRF and IgARF fractions. The RF reactivities were estimated from the differences in absorbance between Fc-coated wells and BSA-coated wells at 415 nm (ΔA_{415}). The specific RF reactivity was indicated as ΔA_{415} per 1 mg protein in each sample. C: Sensorgrams of the IgGRF fractions. Interactions between the isolated IgGRF fractions and immobilized intact human IgG were analyzed at 6°C using the BIAcore X system. All BIAcore manipulations were performed with a continuous flow of HBS at the flow rate of 5 μl/min, as described under "MATERIALS AND METHODS." Total IgG from the pooled sera of the RA patients, or the DE(−)IgG(−)(−)(+), DE(−)IgG(−)(+), DE(−)IgG(+), or DE(−)IgG(−)(−)(−) fraction (5 μg/ml each) was dissolved in HBS and then injected for 7 min over the surfaces of the chips at the flow rate of 5 μl/min.

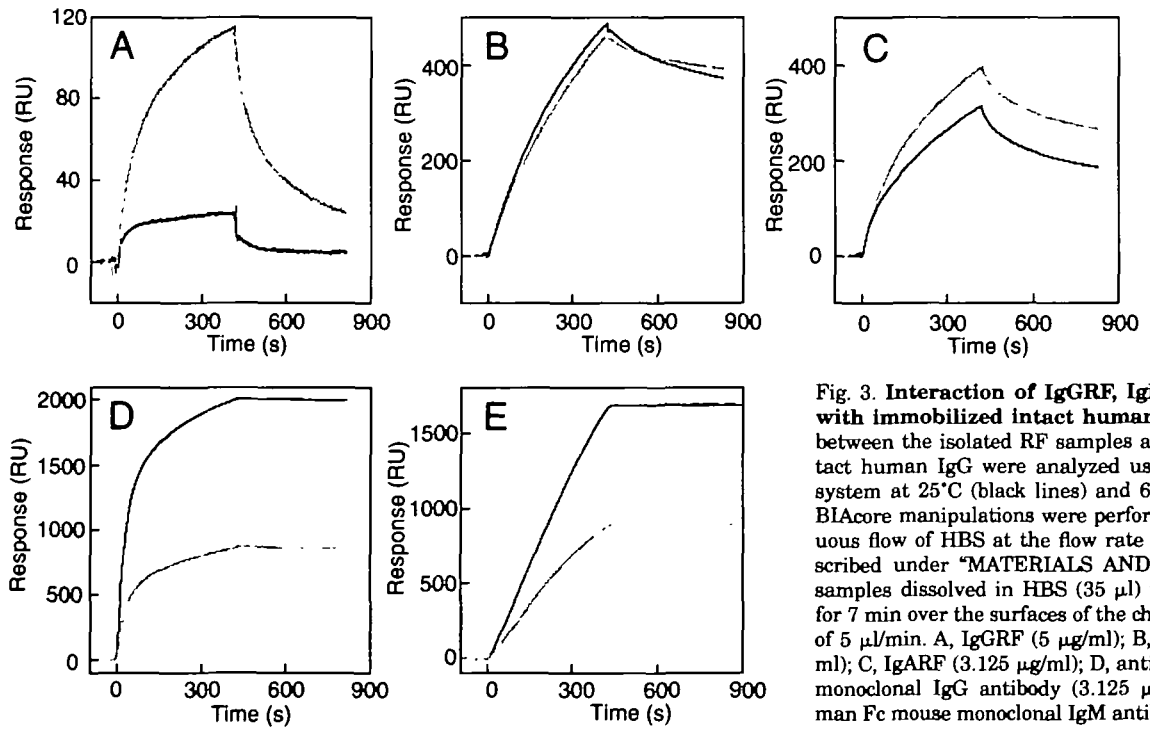


Fig. 3. **Interaction of IgGRF, IgMRF, and IgARF with immobilized intact human IgG.** Interactions between the isolated RF samples and immobilized intact human IgG were analyzed using the BIAcore X system at 25°C (black lines) and 6°C (gray lines). All BIAcore manipulations were performed with a continuous flow of HBS at the flow rate of 5 μl/min, as described under "MATERIALS AND METHODS." The samples dissolved in HBS (35 μl) were then injected for 7 min over the surfaces of the chips at the flow rate of 5 μl/min. A, IgGRF (5 μg/ml); B, IgMRF (3.125 μg/ml); C, IgARF (3.125 μg/ml); D, anti–human Fc mouse monoclonal IgG antibody (3.125 μg/ml); E, anti–human Fc mouse monoclonal IgM antibody (3.125 μg/ml).

than 6°C, *i.e.*, the weaker interaction of IgGRF at 25°C than 6°C was not due to the formation of self-associating IgGRF at 25°C. In addition, the IgGRF which had been eluted from the Superdex 200 HR column at 25°C exhibited almost the same resonance response as that eluted from the column at 4°C (data not shown), suggesting that the tem-

perature dependency of the interaction of IgGRF with IgG was a reversible phenomenon. The interaction of IgARF with the immobilized IgG was only slightly stronger at 6°C than 25°C, but was clearly observed at 25°C (Fig. 3C).

Subsequently, the kinetic parameters of the interactions between RFs and the immobilized IgG were calculated from sensorgrams obtained at 6°C. Table I shows the association rate (k_a), dissociation rate (k_d), and dissociation (K_D) constants of the respective RFs and monoclonal antibodies. In the case of anti-Fc IgM mAb, the k_a and k_d values could not be calculated because dissociation of the antibody from the immobilized IgG was almost undetectable. The K_D value of IgGRF was significantly higher than those of IgMRF, IgARF, and anti-Fc IgG mAb, indicating that the affinity of IgGRF toward IgG was weaker than those of IgMRF, IgARF, and the monoclonal antibody. However, the k_a value of IgGRF (k_a = 1.07 × 10^5 [1/Ms]) was slightly lower than those of the other RF classes or that of the monoclonal antibody (Table I). In contrast, the k_d value of IgGRF (k_d = 2.33 × 10^{-3} [1/s]) was significantly (116- and 15-fold) higher than those of the anti-human Fc mouse monoclonal IgG antibody and IgMRF, and slightly (4.1-fold) higher than that of IgARF. These kinetic parameters obtained at 6°C indicated that the association between IgGRF and the immobilized IgG might occur at a rate similar to in the cases of the other classes of RFs, but that the immune complexes generated in the IgGRF interaction might easily dissociate compared to those involving the other RF classes.

The Effect of Temperature on Interactions between IgG and RFs—Since the interaction between IgGRF and the immobilized IgG was stronger at 6°C than at 25°C, the effect of temperature on this association was compared to that of interactions between other RF classes and human IgG (Fig. 4). IgGRF exhibited almost the same resonance response at 6 and 10°C, but the resonance decreased significantly when the binding assay was carried out at 15°C. At temperatures over 15°C, the resonance units gradually decreased with an increase in temperature (Fig. 4A). At 30°C, the interaction could not be observed by means of surface plasmon resonance (Fig. 4A). In contrast, the interaction of IgMRF with IgG and that of IgARF with IgG showed only

TABLE I. **Kinetic parameters of the interaction of RF with immobilized human IgG at 6°C.** The parameters were calculated from the association and dissociation phases of the sensorgrams of IgGRF, IgMRF, IgARF, the anti-Fc mouse monoclonal IgG antibody (anti-Fc IgG mAb), and the anti-Fc mouse monoclonal IgM antibody (anti-Fc IgM mAb), as described under "MATERIALS AND METHODS."

	k_a(1/Ms) (× 10^5)	k_d(1/s) (× 10^{-4})	K_D(M) (× 10^{-10})
IgGRF	1.07	23.3	218
IgMRF	5.15	1.59	3.09
IgARF	2.63	6.58	25.0
Anti-Fc IgG mAb	4.93	0.201	0.408
Anti-Fc IgM mAb	N.C.	N.C.	N.C.

N.C., could not be calculated.

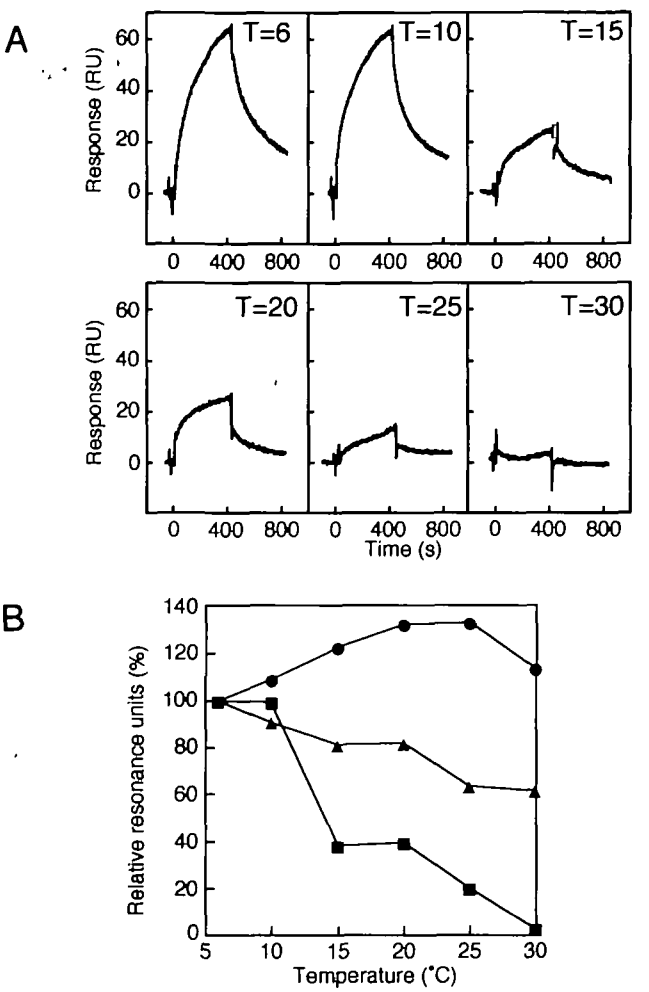


Fig. 4. **Effect of temperature on the interaction between IgGRF and immobilized human IgG.** A: Interaction between IgGRF (3.125 μg/ml) and immobilized intact human IgG was analyzed using the BIAcore X system at 6, 10, 15, 20, 25, and 30°C. B: Relationship between temperature and the resonance response exhibited by the purified RF. The resonance response exhibited by purified IgGRF (■), IgMRF (●), or IgARF (▲) at each temperature was estimated from the average resonance units between 393 and 402 s in each sensorgram. The ordinate represents the relative resonance units calculated from the resonance units of each RF sample at 6°C.

TABLE II. **Kinetic parameters of RFs at different temperatures.** The parameters were calculated from the association and dissociation phases of the sensorgrams, as described under "MATERIALS AND METHODS."

Temperature (°C)	IgGRF		IgMRF		IgARF	
	k_a (1/Ms) (× 10^4)	k_d (1/s) (× 10^{-4})	k_a (1/Ms) (× 10^4)	k_d (1/s) (× 10^{-4})	k_a (1/Ms) (× 10^4)	k_d (1/s) (× 10^{-4})
6	10.70	23.3	51.5	1.59	26.3	6.58
10	8.08	21.2	57.7	1.59	23.5	7.79
15	2.36	21.1	67.3	2.01	19.5	8.44
20	1.88	23.8	73.7	2.37	21.1	9.49
25	0.95	6.1	77.0	3.41	14.6	8.73
30	N.C.	N.C.	63.1	4.95	12.9	10.50

N.C., could not be calculated.

a weak temperature dependency. The resonance response exhibited by IgMRF at 30°C was higher than that at 6°C (Fig. 4B). IgARF clearly interacted with the immobilized IgG, even at 30°C, although the interaction at 30°C was slightly weaker than that at 6°C (Fig. 4B). These results indicated that the temperature dependency of the interaction between IgGRF and human IgG differed completely from those in the cases of IgMRF and IgARF.

The k_a and k_d values of the RFs at each temperature were then calculated from the sensorgrams (Table II). The k_a values of IgGRF were similar at 6 and 10°C, but it was greatly reduced at 15°C and decreased gradually with temperature over 15°C (Table II). At 25°C, this value was over 10-fold less than it was at 6°C. In contrast, the k_d values of IgGRF at 6, 10, 15, and 20°C were almost the same (about 2×10^{-3} [1/s]), but this value was distinctly reduced at 25°C (Table II). It should be noted that the k_d value of IgGRF at 25°C is similar to those of IgMRF and IgARF at 25°C (Table II). The k_a and k_d values of IgGRF at 30°C could not be calculated because the interaction was almost undetectable. As for other RFs, the k_a values of IgARF decreased only slightly with an increase in the assay temperature (Table II) and those of IgMRF were not significantly different at the different temperatures. In addition, the k_a value of the anti–Fc IgG mAb did not change (data not shown). On the other hand, the k_d values of IgARF and IgMRF increased slightly and gradually with an increase in the assay temperature (Table II). Therefore, the mode of interaction between IgGRF and immobilized IgG differed from those in the cases of other classes of RFs.

DISCUSSION

Rheumatoid factors (RFs) are autoantibodies characteristically associated with rheumatoid arthritis. These factors recognize antigens on the Fc region of IgGs. RFs can exist as any of the immunoglobulin isotypes, but among the various RF classes, IgGRF has been implicated in the pathogenesis of RA since it forms large immune complexes in rheumatoid joints (*2*, *5*, *6*). In the present study, to characterize the formation of these IgGRF complexes, we examined the interaction of IgGRF with IgG and compared it to those in the cases of other RF classes by means of surface plasmon resonance (*12*, *13*). We demonstrated that IgGRF was more reactive with immobilized IgG at lower temperatures, while the reactivities of other classes of RFs were hardly affected by any change in the thermal conditions.

In order to analyze the interaction between RFs and IgG, we successfully purified IgGRF, IgMRF, and IgARF from pooled sera of RA patients by means of IgG-Sepharose affinity chromatography after first separating the IgG from other classes of immunoglobulin by DEAE-cellulose column chromatography. It is especially interesting that the isolated IgGRF could hardly form self-aggregating complexes in solution, as demonstrated by the fact that the IgGRF sample was eluted as a single peak corresponding to monomeric IgG on a Superdex 200 HR column (Fig. 1B). In addition, the isolated IgM and IgA samples contained only IgM and IgA, respectively, and were not contaminated by IgG (Fig. 1D). Based on these results, we judged that IgG-containing immune complexes were negligible in the IgGRF, IgMRF, and IgARF samples prior to the interaction assay. We then analyzed the interactions of these RF samples with immobilized IgG by means of surface plasmon resonance. The resonance response levels exhibited by the three IgGRFs increased relative to the strength of each specific RF reactivity, as detected on ELISA, indicating that surface plasmon resonance is useful for analyzing the formation of IgGRF immune complexes (Fig. 2). The interaction of IgGRF with immobilized IgG was clearly observed by means of surface plasmon resonance. In addition, IgG-Sepharose affinity chromatography as well as ELISA showed that IgGRF molecules avidly bound to IgG or Fc immobilized on the solid phase. However, the IgGRF from RA patients apparently failed to efficiently form IgG-IgG self-aggregating complexes in solution. One possible reason for this was the use of a relatively low concentration of IgGRF in the sample (145 μg/ml) employed under our experimental conditions.

With surface plasmon resonance detection, we found that the interaction between IgGRF and immobilized IgG decreased inversely with an increase in temperature. The interaction was clearly observed at 6°C but could not be detected at 30°C. In contrast, IgMRF and IgARF strongly interacted with the immobilized human IgG at both 30 and 6°C. Therefore, the effect of temperature on the interaction of IgGRF with immobilized IgG differed from in the cases of IgMRF or IgARF. The dissociation constant (K_D) for the IgGRF interaction with human IgG indicates that the affinity between IgGRF and IgG is very weak compared to that in the cases of other classes of RFs or the anti-Fc monoclonal antibodies. However, in order to gain an understanding of interactions *in vivo*, association rate (k_a) and dissociation rate (k_d) constants would be more important than K_D values, since K_D values only reflect affinity in the equilibrium state. It should be noted that at 6°C the association rate constant (k_a) of the IgGRF interaction with human IgG was similar to those in the cases of the other RF classes. It clearly decreased with an increase in the assay temperature, while those of IgARF, IgMRF, and the anti-Fc monoclonal IgG antibody exhibited similar values under different thermal conditions. In contrast, the dissociation rate constants (k_d) of IgARF and IgMRF increased slightly and gradually with an increase in assay temperature. However, in the case of IgGRF, the k_d value remained unchanged from 6 to 20°C, but was greatly reduced at 25°C. These results clearly demonstrated that k_a values were strongly affected by temperature in the case of the IgGRF-IgG interaction, while k_d values rather than k_a values were slightly affected in the case of interaction of other RF classes with IgG. Therefore, the interaction between IgGRF and immobilized IgG could be distinguished kinetically from those in the cases of other classes of RFs. These results indicate that the mechanism of formation of immune complexes involving IgGRF may differ from in the cases of those involving other RF classes. It was earlier suggested that Fab oligosaccharides may be inserted into the vacant pocket of Fc, resulting in the formation of IgG-IgG complexes, which did not require an actual antigen–antibody interaction (*15*, *16*). These pockets are considered to be formed due to a deficiency in galactose on Fc oligosaccharides. However, when all the galactose residues were enzymatically cleaved from IgG molecules derived from healthy individuals, there was no detectable binding of the resulting galactose-free IgG or the intact IgG with immobilized IgG (unpublished observation). Therefore, the interaction between IgGRF and immo-

bilized IgG most probably occurred as an actual antigen-antibody association.

Our results may increase the understanding of the formation of IgGRF immune complexes in rheumatoid joints. We have shown here that IgGRF was more reactive to immobilized IgG at a lower temperature, indicating that IgGRF forms immune complexes much more easily when the temperature is low. The more important point, however, is that the association rate constant (k_a) of IgGRF exhibited a value at 6 or 10°C similar to those of other classes of RFs as well as to that of the anti-Fc monoclonal IgG antibody. This suggested that the formation of IgGRF immune complexes might occur at a rate similar to in the cases of other RF classes. In addition, the dissociation rate constant (k_d) for the dissociation of IgGRF from the immobilized IgG was greatly reduced at over 25°C. The k_d value of IgGRF at 25°C is comparable to those of IgMRF and IgARF (Table II). These kinetic properties of the IgGRF–IgG interaction suggest that the formation of immune complexes involving IgGRF can be accelerated by a significant change in temperature, *i.e.*, IgGRF immune complexes once formed at lower temperatures may be stabilized (or difficult to dissociate) at higher temperatures. Since IgGRF is the most abundantly synthesized antibody in the rheumatoid synovium (*5*) and a decrease in temperature has been shown to be associated with disease activity in RA (*17*, *18*), our findings may help in clarifying the relationship between IgGRF and the disease activity and pathogenesis of RA.

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