Distribution-Analyzing Latex Immunoassay (DALIA): Methods for Determination of Antigen and for Elimination of Non-specific Reaction Induced by Rheumatoid Factor

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A highly sensitive distribution-analyzing latex immunoassay method (DALIA), which is based on analysis of the volume distribution of latex particles including both the agglutinates and the residual non-agglutinating particles, has been established. Numbers of latex particles, which are sensitized with specific antibodies, are counted by using an electric particle counter with a personal computer and simultaneously the extent of agglutination was quantified by analyzing the volume distribution of the reacted latex particles. It was found that ultramicrospheres coated with normal goat immunoglobulin G can completely eliminate non-specific reactions induced by human serum containing rheumatoid factor (RF) in this DALIA method. The degree of absorption of RF activity by these ultramicrospheres was associated with the diameters of the ultramicrospheres. Moreover, by use of the combination of ultramicrospheres and latex particles coated with monoclonal antibodies against α-fetoprotein (AFP) or with polyclonal antibody specific to C-reactive protein, specific DALIA systems were able to be developed. Both DALIA systems exhibited the high sensitivity (1 to 7 ng/ml). The correlation coefficient (γ) of the results of DALIA with those of enzyme-linked immunoadsorbent assay in measuring AFP was 0.994.

Keywords latex; immunoassay; rheumatoid factor; ultramicrosphere; antigen

Many immunoassay systems employing latex particles sensitized with antigen or antibody specific to antigen and utilizing by physicochemical binding or spontaneous adsorption or physical adsorption methods have been reported.[1)] In order to achieve precise determination of trace materials in patient's sera (for example, a tumor marker, hormone or coagulation factor), it is important to detect these materials specifically without any influence of factors such as rheumatoid factor (RF), which are known to induce a non-specific reaction. Attempts have been made to avoid the non-specific reaction induced by RF, by the use of latex particles sensitized with $F(ab')_2$ fragments[1d)] or by treatment with a reducing agent such as dithiothreitol (DTT)[2)] or 2-mercaptoethanol.[3)] However, it is well known that RF also exists in the immunoglobulin G (IgG) class, although it is dominantly in the IgM,[4)] and that there is a similarity in patterns of isotype distribution of anti-$F(ab')_2$ antibodies and RF.[5)] Therefore, it is important to eliminate completely the participation of such RFs, especially in order to detect low concentrations of antigens and antibodies in human serum.

Recently, a sensitive and specific latex immunoassay (PACIA) based on counting the residual non-agglutinating latex particles has been reported.[6)] In contrast to the PACIA method counting the absolute numbers of the residual non-agglutinating latex particles in a reaction mixture, we have established a sensitive latex immunoassay, the distribution-analyzing latex immunoassay (DALIA), which is based on analysis of the volume distribution of latex particles including both the agglutinates and the residual non-agglutinating particles. We found that ultramicrospheres coated with normal IgG can inhibit non-specific reactions more effectively than aggregated IgG. Therefore, this method for more effectively eliminating the non-specific reaction induced by RF was applied to the DALIA system.

Materials and Methods

Buffer Solutions (a) GB is a glycine buffer solution containing, in each liter, 3.75 g of glycine and 1 g of NaN_3, and adjusted to pH 8.0 with 5 N NaOH. (b) GB–BSA is GB containing, in each liter, 2 to 5 g of bovine serum albumin (BSA) (A-7906, lot 113F-0123; Sigma). (c) GBS–BSA is GB containing, in each liter, 8.5 g of NaCl and 2 g of BSA. (d) GB–BSA–T80, which is used as the aging buffer solution for antibody-sensitized latex, contained 0.2 g/l Tween 80 (Daiichi Pure Chemicals, Co.) in GB–BSA. All the buffer solutions described above were filtered through a 0.22 μm membrane filter (GSWP04700; Millipore Ltd.).

Patients' and Normal Human Sera (NHS) NHS were obtained from volunteers in our laboratory after an overnight fast. Pathological human sera from patients suffering from rheumatoid arthritis (RA) were kindly supplied by Dr. K. Inagaki (Inagaki Clinic, Tokyo). Some of these RA sera were pooled and used as material for purification of RF. The pooled RA sera and RF purified from these sera had activity even when treated with 5 mM DTT, so that they also contained IgG-RF resistant to the treatment with DTT. In fact, the presence of IgG-RF was revealed by a check of the purity, by 4—20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). The RF activities of these RA sera and purified RF were measured by the passive hemagglutination method for RF.

IgG and $F(ab')_2$ $F(ab')_2$ fraction of sheep anti-C-reactive protein (CRP) antiserum was purchased from Cappel (Cooper Biomedical, Inc.) and monoclonal antibody against human α-fetoprotein (AFP) was prepared in our laboratory.[7)] IgG fractions of normal goat, rabbit, mouse, bovine, and horse serum were purified with DEAE-Sephacel (Pharmacia, Uppsala; column size 100 × 4.5 cm, 0.05 M sodium phosphate buffer solution, pH 8.0) chromatography. $F(ab')_2$ fragments of normal goat IgG and of monoclonal antibodies were prepared by pepsin digestion[8)] and by partial purification with Sephadex G-100 or DEAE-Sephacel (Pharmacia, Uppsala). Aggregated IgG from normal goat serum was prepared by heating IgG at 63 °C for 10 min.[9)]

Ultramicrosphere Preparation Aliquots of 0.1 ml of polystyrene latex (100 g/l) with diameters (d) of 0.089, 0.2 μm (Estapor K587, Rhone-Poulenc, Courbevoie) and 0.109 μm (DOW Chemicals) were each mixed with 10 ml of purified IgG (5 mg/ml, GB) of normal serum from goat, rabbit, mouse, bovine and horse. After incubation for 30 min, 10 ml of GB–BSA–T80 was added to the obtained mixture in order to avoid spontaneous aggregation of the coated latex. The resultant suspension was applied to a Sepharose 4B (Pharmacia, Uppsala; column size 100 × 4.5 cm, GB–BSA) column to eliminate non-adsorbed IgG and the void fraction was collected. Then the particle sizes and distributions of freshly prepared ultramicrospheres and also of the aggregated ultramicrospheres were analyzed by means of photon correlation spectroscopy (Particle Sizer, model BI-90, Brookhaven Instruments Co.).[10)]

Antibody-Coated Latex (a) $F(ab')_2$ fragments of each monoclonal antibody (clone Nos. 02205 and 02211; 100 μg/ml; 0.05 M GB, pH 8.0) against AFP were mixed with an equal volume of 1% (w/v) latex suspension (SFL140S9 and SFL140S3, 0.8 μm diameter; Japan Synthetic Rubber Co., Ltd., Japan) and incubated at 4 °C for 2 h. Then the latex suspension was washed by centrifugation (× 10000 rpm, 20 min) with GB–

BSA–T80 (2 vol.) and resuspended in the same buffer. The resulting suspension was incubated at 37 °C for 2 h, washed by repeated centrifugation (×12000 rpm, 20 min), and resuspended to a 0.5% latex concentration in GB–BSA. Finally, the two species of monoclonal antibody-coated latex particles were mixed. (b) $F(ab')_2$ fragments of sheep anti-CRP antibody (500 μg/ml; 0.1 M GB, pH 8.0) were mixed with an equal volume of latex suspension (10 g/l, $d=0.804$ μm; Estapor K109; Rhone-Poulenc, Courbevoie). After incubation at 4 °C for 2 h, the obtained latex suspension was treated in the same manner as the monoclonal antibody-coated latex suspension.

RF Purification RF was prepared by a specific immunoadsorption method. Briefly, 30 ml of pooled serum from patients showing classical RA was dialyzed at 4 °C against cold distilled water. The obtained euglobulin fraction was dissolved in 0.05 M phosphate buffer solution in saline (PBS), pH 7.4, and dialyzed against same buffer solution. The euglobulin fraction was further purified by specific adsorption on a homologous IgG affinity column with 7 ml of CNBr-activated Sepharose 4B coupled with 200 mg of human IgG (Cappel, Inc.). The column was then washed with PBS containing 0.5 M NaCl and the bound RF was eluted with a 0.1 M glycine–HCl buffer solution, pH 2.8. After immediate neutralization with 2 M Tris, each purified RF was dialyzed against PBS and the purity was checked by 4 to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis[1]; IgM- and IgG-RF were detected. The RF activity was determined by the hemagglutination method. The RF titer was 1/1280 and the activity was partly resistant to treatment with 1.3—5 mM DTT.[2]

DALIA For the DALIA system, 50 μl of standard solution or human serum after suitable dilution with GBS–BSA–T80 or the ultramicrosphere suspension was mixed with 50 μl of 0.1 to 0.5% antibody- or normal IgG-coated latex particles. After continuous incubation for 10 or 20 min at 37 °C, the mixed suspension was diluted 200- to 800-fold to stop the immunoreaction, and then the particle volume distribution of the diluted latex suspension containing both non-agglutinating particles and the agglutinates were measured by a Coulter Counter Model ZBI & C-1000 Chanalyzer (Coulter Electronics, Inc.), employing a 30 μm aperture tube, which is based on the Coulter principle. Particle numbers per volume (μm^3) were counted by the counter system and the obtained data were transferred to a personal computer (NEC PC-8801) connected with the C-1000 Chanalyzer *via* an interface. The transferred data (volume distribution of latex particles) were analyzed and processed in the computer with original software. As shown in Fig. 1a, only the first peak was recognized when an antigen- or antibody-free sample was used as a control. On the contrary, with increasing concentration of antigen or antibody in human samples or standard solution, the agglutinates indicated as the second peak and above in Fig. 1b and c, were increased, where the second and third peaks correspond to the dimer and trimer of latex particles, respectively. Therefore, the extent of agglutination was evaluated in terms of integral volumes (*TV*; μm^3), obtained by multiplying the particle numbers by the volumes of agglutinates, with respect to a constant number (20000 counts) of residual nonagglutinating particles (first peak of Fig. 1). The actual particle distribution and the data obtained by the DALIA system are shown in Fig. 2. CRP standard serum (7.6 mg/ml) was purchased from Behringwerke AG.

Results

Absorption of RF Activity The ability of various forms of normal goat IgG to absorb RF activity was checked by measuring the inhibition of agglutination of latex particles coated with normal goat IgG or $F(ab')_2$ fragment of sheep anti-CRP antibody, induced with RF (Table I or II). The extent of agglutination (*TV*) of latex particles was measured by the DALIA system, and the extent of absorption activity with the ultramicrosphere suspension, normal IgG, $F(ab')_2$ or heat-aggregated IgG was expressed as a percentage in comparison with the *TV* of agglutination of the positive control induced with RF. The maximum agglutination of both latex particles (control in Tables I and II) induced with RF was completely reduced or inhibited by pretreatment of RF with the ultramicrosphere suspension ($d=0.089$ μm) even when the IgG concentration (0.8 mg/ml) on the surface of the ultramicrospheres responsible for absorption of RF was lower than the concentration of normal IgG or $F(ab')_2$ (10 mg/ml). On the other hand, the absorption activity of the heat-aggregated IgG (10 mg/ml) to inhibit positive agglutination induced with RF was lower than the activity of the ultramicrosphere suspension. Furthermore, the ultramicrospheres coated with normal goat IgG were also able to inhibit the non-specific agglutination of the latex particles coated with $F(ab')_2$ fragments of anti-CRP antibody (Table II). The present capability of the ultramicrospheres for eliminating the nonspecific agglutination induced by RF was confirmed by the experiment using

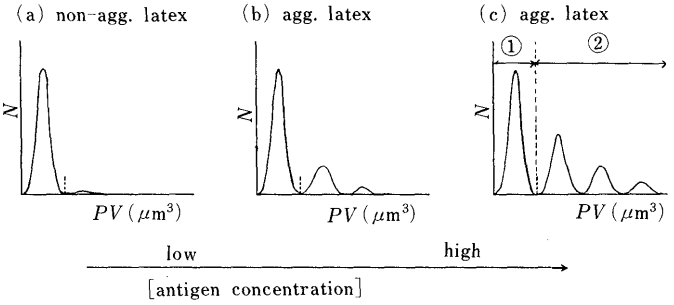

Fig. 1. Principle of the DALIA System

Agg., agglutinated; *N*, number; *PV*, particle volume.

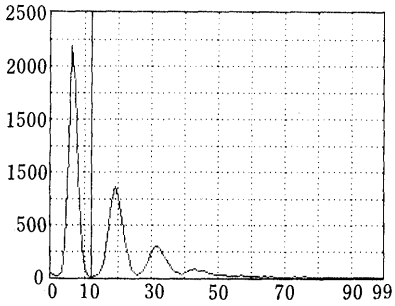

Fig. 2. Data Print-out from the DALIA System

Analysis of distribution: total count (0—99 channel): 14239. cut-off channel: 1.

	[1st peak]	[agglutinates]
peak range	2—10 (6)	11—99
count	6998	7251
volume (μm^3)	4056.8	11025.3

These data were obtained by analysis of latex particles coated with anti-CRP·$F(ab')_2$ antibody.

TABLE I. Comparison of Absorption Activity with Various Forms of IgG

IgG form for RF absorption[a]	Concentration (mg/ml)	Total volume (μm^3)	Absorption activity (%)[c]
Buffer solution (control)	—	31850±2803[b]	—
$F(ab')_2$	10	31085±2653	2.4
IgG	10	30066±2088	5.6
Heat-aggregated IgG	10	7323± 842	83.0
	1	13031±1329	63.0
Ultramicrosphere	0.8[d]	159± 23	99.5

a) RF was reacted with the goat normal IgG-coated latex particles (0.805 μm) after 20-fold dilution with buffer, $F(ab')_2$, normal IgG, heat-aggregated IgG or ultramicrospheres. *b*) Mean±S.D. ($n=4$). *c*) Calculated by using the following equation: $\frac{TV \text{ of control} - TV \text{ of various IgG forms}}{TV \text{ of control}} \times 100(\%)$, where *TV* means total volume. *d*) The IgG concentration on the surface of the ultramicrospheres.

Table II. Comparison of Absorption Activity with Various Forms of IgG

IgG form for RF absorption[a]	Concentration (mg/ml)	Total volume (μm^3)	Absorption activity (%)[c]
Buffer solution (control)	—	22323±1392[b]	—
$F(ab')_2$	10	20351±1154	8.8
IgG	10	21274±1038	4.7
Heat-aggregated IgG	10	12446± 553	44.3
Ultramicrosphere	1	—	
	0.8[d]	187± 34	99.2

a) RF was reacted with the latex particles (0.805 μm) coated with $F(ab')_2$ fragment of sheep anti-CRP antibody (0.805 μm) after 20-fold dilution with buffer solution, $F(ab')_2$, normal IgG, heat-aggregated IgG or ultramicrospheres. *b*) Mean±S.D. ($n=4$). *c*) Calculated by using the following equation: $\frac{TV \text{ of control} - TV \text{ of various IgG forms}}{TV \text{ of control}} \times 100(\%)$, where TV means total volume. *d*) The IgG concentration on the surface of the ultramicrospheres.

Table III. Comparison of Absorption Activity with IgG from Various Species

Species of purified IgG (ultramicrospheres)[a]	Total volume (μm^3)	Absorption activity[c] (%)
Buffer solution (control)	31079±2650[b]	—
Goat IgG	251± 24	99.2
	322± 28	99.0
Rabbit IgG	415± 98	98.7
Bovine IgG	1485± 131	95.2
Horse IgG	—[d]	—[d]
Mouse IgG	6297± 281	79.3

a) RF was diluted 20-fold with buffer solution, ultramicrospheres coated with normal IgG of various species and then reacted with the goat normal IgG-coated latex particles (0.805 μm). *b*) Mean±S.D. ($n=4$). *c*) Calculated by using the following equation: $\frac{TV \text{ of control} - TV \text{ of various IgG forms}}{TV \text{ of control}} \times 100(\%)$, where TV means total volume. *d*) A precise counting was not possible because of marked spontaneous agglutination.

Table IV. Effect of Diameter of Ultramicrospheres on RF-Absorption Activity

Diameter of ultramicrospheres[a]	Total volume (μm^3)	Absorption activity[c] (%)
Buffer solution (control)	29439±2650[b]	—
0.089 μm	118± 24	99.6
0.109 μm	1236± 98	95.8
0.2 μm	U.C.[d]	U.C.

a) RF was diluted 20-fold with buffer or ultramicrospheres, and then reacted with the goat normal IgG-coated latex particles (0.805 μm). *b*) Mean±S.D. ($n=4$). *c*) Calculated by using the following equation: $\frac{TV \text{ of control} - TV \text{ of various IgG forms}}{TV \text{ of control}} \times 100(\%)$, where TV means total volume. *d*) U.C.=uncountable. A precise counting was impossible although the test was done.

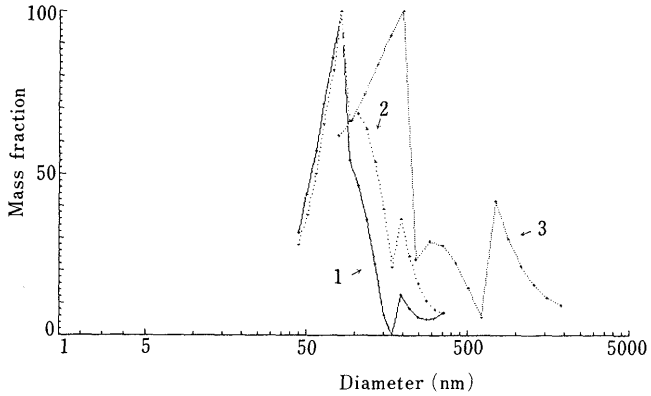

Fig. 3. Analysis of Particle Distribution of the Ultramicrospheres by Photon Correlation Spectroscopy

1, —— 0.089 μm+RF; 2, ···· 0.109 μm+RF; 3, ····· 0.2 μm+RF.

After reaction with RF (20-fold dilution), the particle size and distribution of ultramicrospheres (0.1%) were estimated.

individual RF positive sera from RA patients ($n \geqq 130$) instead of the purified RF, although the data are not shown.

Effect of IgG Species In order to check the difference of absorption ability of IgG among different species, ultramicrospheres with a diameter of 0.089 μm were sensitized with normal IgG purified from goat, rabbit, mouse, bovine or horse serum, and their RF-absorbing ability was studied by the same DALIA system with normal IgG-coated latex particles described previously.

As shown in Table III, the ultramicrospheres coated with various normal IgG (from goat, rabbit, or bovine serum) strongly inhibited the non-specific agglutination induced by RF, except for ultramicrospheres coated with mouse IgG. However, the horse IgG-coated ultramicrospheres were unsuitable because of spontaneous agglutination.

Diameter of Ultramicrospheres As indicated from the data of the inhibition experiments with the ultramicrosphere suspension, IgG adsorbed on the surface of latex particles showed enhanced absorption of RF activity. It should be considered that the marked potentiation of the ultramicrospheres on the RF-absorption activity depended primarily on the very large specific surface area coated with normal IgG, compared with that of the latex particles with a diameter of 0.8 μm. So, the influence of the diameter of the ultramicrospheres on the absorption activity of RF was studied using three kinds of ultramicrospheres with a diameter of 0.089, 0.109 or 0.2 μm (Table IV).

As shown in Table IV, the absorption ability (99.6%) of ultramicrospheres with a diameter of 0.089 μm was comparatively greater than that (95.8%) of 0.109 μm ultramicrospheres at the same concentration, although both ultramicrospheres exhibited a sufficient RF-absorption activity. On the other hand, in the case of ultramicrospheres with a diameter of 0.2 μm, a precise counting of normal IgG-coated latex particles ($d=0.805$ μm) was not possible because of the interference by aggregates of 0.2 μm ultramicrospheres with the counting of latex particles.

The reason why the ultramicrosphere suspension had no influence on the counting of latex particles ($d=0.805$ μm) may be as follows. Due to the smallness of the initial ultramicrospheres, the volume (μm^3) of the 0.089 and 0.109 μm ultramicrosphere did not approach that of latex particles ($d=0.805$ μm), even if the ultramicrospheres were heavily aggregated by RF. Therefore, a precise counting of the latex particles with a diameter of 0.805 μm is possible. This conclusion was supported by analysis of the particle size and distribution of the three kinds of ultramicrospheres after reaction with RF by photon correlation spectroscopy (Fig. 3). However, the use of 0.2 μm ultramicrospheres, which have about eleven-fold more volume compared with

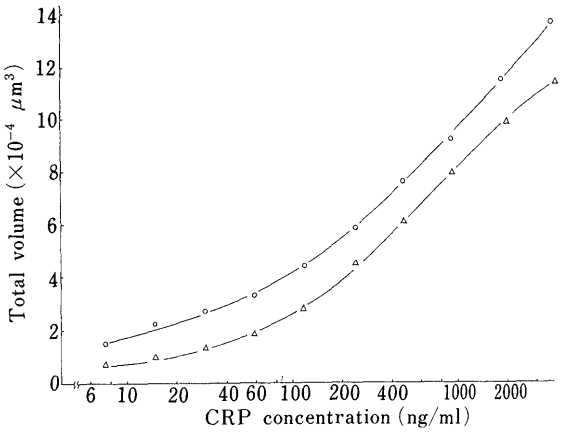

Fig. 4. Standard Curves for the Quantitation of CRP by DALIA with Anti-CRP·F(ab')$_2$-coated Latex Particles

Standard solution of CRP was diluted with: ○, GB–BSA–T80 or △, ultramicrosphere suspension.

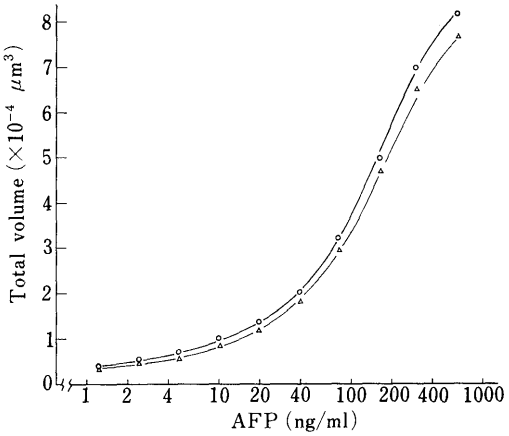

Fig. 5. Standard Curves for the Quantitation of AFP by DALIA with Anti-AFP Monoclonal Antibody-coated Latex Particles

Standard solution of AFP was diluted with :○, GB–BSA–T80 or △, ultramicrosphere suspension.

0.089 μm ultramicrosphere, was not practical because the increased volume of the resultant aggregates was greater than that of latex particles (0.805 μm) after absorption of RF.

CRP-, AFP-DALIA We attempted to apply the ultramicrospheres to the DALIA system in order to measure the CRP and AFP value in human sera, and the influence of the ultramicrospheres on the standard curve was studied (Figs. 4 and 5).

A 50 μl aliquot of standard solution of CRP and AFP diluted with GB–BSA–T80 or ultramicrospheres (d= 0.089 μm) was mixed with 0.2% sheep anti-CRP·F(ab')$_2$-coated latex particles and with 0.5% anti-AFP monoclonal antibodies-coated latex particles, respectively, and the mixtures were reacted at room temperature for 10 and 20 min. After suitable dilution to stop the immunoreaction, the extent of the agglutination (TV) was determined. CRP standard serum (7.6 mg/ml) was purchased from Behringwerke AG.

It seemed that the degree of the immunoreaction obtained in the DALIA system with the ultramicrospheres was slightly decreased compared with that of the usual DALIA system without the ultramicrospheres. But both

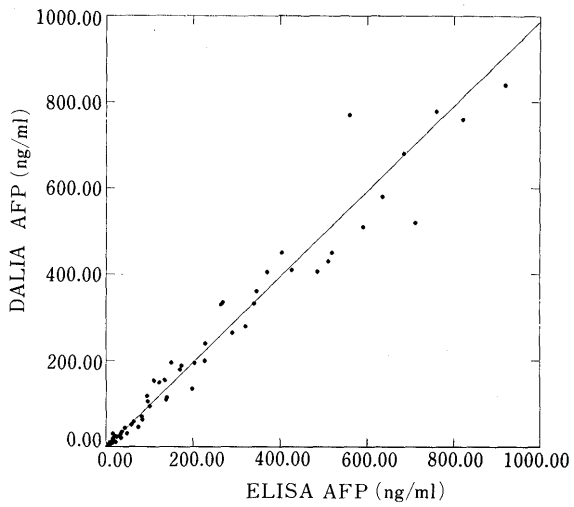

Fig. 6. Correlation between the Results of Determination of AFP by ELISA or by DALIA

$N=64$, $Y=0.991X-2.876$, $\gamma=0.994$.

DALIA systems exhibited high sensitivity (1 to 7 ng/ml) in the measurement of CRP and of AFP, and good reproducibility (coefficient of variation; $CV=2.3-7.9\%$) was obtained (data not shown).

Comparison with Enzyme-Linked Immunoadsorbent Assay (ELISA) For the determination of AFP by DALIA, a 50 μl aliquot of serum sample diluted 1 : 10 with the ultramicrospheres ($d=0.089$ μm) was mixed with 50 μl of the monoclonal antibody-coated latex particles and reacted at 37 °C for 20 min.

Sixty-four serum samples from patients were assayed in duplicate by the DALIA system, and the means of the two results were compared with those obtained with the ELISA[7] which was established in our laboratory. The correlation between the results obtained by DALIA and by ELISA is shown in Fig. 6. There were no systematic differences between the two methods in the high and low concentration ranges and a good correlation coefficient ($\gamma=$ 0.994) was obtained.

Discussion

By using the method of analyzing the volume distribution of latex particles including both the agglutinates and the residual non-agglutinating particles, classified by particle diameter, we have developed a highly sensitive latex immunoassay method (DALIA method). As a practical assay system, DALIA is sufficiently sensitive and simple. The assay itself is very rapid, requiring an incubation time for only 10 or 20 min followed by measurement of the agglutination. It should be noted that a prolongation of the reaction time, for example 30 to 45 min, can increase the sensitivity, although the detection limit of CRP and AFP on the present study was 1 to 7 ng/ml. Extensive studies on precision and accuracy are now being performed for the determination of IgE, AFP, cross-linked fibrin degradation products (FDP), β-human chorionic gonadotropin and so on. From preliminary results, we are able to report that the CV values of ten repeated determinations of antigens in plasma samples were less than 5%.

For the determination of AFP and CRP, Collet-Cassart *et al.*[6b,12] reported a highly sensitive latex immunoassay

(PACIA) based on counting of residual nonagglutinating particles. In their work, latex particles coated with the $F(ab')_2$ fragments of the immunoglobulin G fraction of the antiserum were used to avoid interference by serum constituents. At the same time, they found a significant agglutinating activity toward $F(ab')_2$ fragments of non-immunized rabbit in a few patients' sera. The agglutinators which exhibited anti-$F(ab')_2$ activity could be absorbed on aggregated $F(ab')_2$ fragments from non-immunized rabbit. We were also able to detect a similar activity against $F(ab')_2$ fragments in the experiment with the purified RF and the latex particles coated with $F(ab')_2$ fragments of rabbit anti-CRP antibody. However, the nonspecific agglutination depending on such an anti-$F(ab')_2$ activity was also eliminated by using the ultramicrospheres coated with normal IgG from rabbit and goat serum.

The method for elimination of the non-specific reaction is in principle supported by the facts that the ultramicrospheres can exhibit high potentiation of the absorption of RF compared with the aggregated IgG. The capability of the ultramicrospheres for absorbing RF activity appears to correlate with the very large comparative specific surface area and perhaps the energy of the Brownian motion depending on the very small volume of the particles compared with antibody-coated latex. Therefore, with increasing diameter of the ultramicrospheres, it seems that the absorption activity tended to decrease, and finally it became impossible to count agglutinated antibody-coated latex particles ($d=0.8\,\mu$m) due to interference by the aggregated ultramicrospheres.

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