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Abstract

We have already developed the liposome immune lysis assay (LILA) for the determination of C-reactive protein (CRP) by employing an inhibition method and a sandwich method. We herein report a new LILA system involving the use of monoclonal antibodies-bearing liposomes. We established five monoclonal antibodies to CRP antigen, AC-1, -2, -3, -4, -5 which had the capacity to activate complement and form antigen-antibody complex. Each of these antibodies was covalently coupled to carboxyfluorescein-entrapped multilamellar liposomes. When the liposomes were incubated with CRP antigen in the presence of guinea pig complement, CRP antigen-dependent liposome lysis was observed but the sensitivity was not great enough for practical use. On the other hand, when liposomes coupling two monoclonal antibodies (AC-1, AC-2) which recognized distinct CRP antigenic determinants were employed in the assay, the sensitivity increased compared with that using only one monoclonal antibody, and the detectable concentration range was 5-300 ng/ml. These results indicated that the combination of two or more monoclonal antibodies which recognize distinct CRP antigenic determinants is effective for increasing the sensitivity of the assay.

KEYWORDS: liposome immune lysis assay, C-reactive protein, carboxyfluoescein, mouse monoclonal antibodies

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A Novel Liposome Immune Lysis Assay (LILA) for Determination of CRP Antigen Using Two Monoclonal Antibodies Recognizing Different Antigenic Determinants

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We have already developed the liposome immune lysis assay (LILA) for the determination of C-reactive protein (CRP) by employing an inhibition method and a sandwich method. We herein report a new LILA system involving the use of monoclonal antibodies-bearing liposomes. We established five monoclonal antibodies to CRP antigen, AC-1, -2, -3, -4, -5 which had the capacity to activate complement and form antigen-antibody complex. Each of these antibodies was covalently coupled to carboxyfluorescein-entrapped multilamellar liposomes. When the liposomes were incubated with CRP antigen in the presence of guinea pig complement, CRP antigen-dependent liposome lysis was observed but the sensitivity was not great enough for practical use. On the other hand, when liposomes coupling two monoclonal antibodies (AC-1, AC-2) which recognized distinct CRP antigenic determinants were employed in the assay, the sensitivity increased compared with that using only one monoclonal antibody, and the detectable concentration range was 5-300 ng/ml. These results indicated that the combination of two or more monoclonal antibodies which recognize distinct CRP antigenic determinants is effective for increasing the sensitivity of the assay.

Key words: liposome immune lysis assay, C-reactive protein, carboxyfluorescein, mouse monoclonal antibodies

Liposomes have been widely applied in the field of biology and medicine (1). We have also developed the complement-dependent liposome immune lysis assay (LILA) using carboxyfluorescein (CF)-entrapped liposomes bearing antigens or antibodies as a diagnostic tool (2-5). This assay system is based on the fact that CF acts as a self-quenching fluorophore when encapsulated in liposomes at high concentrations and that it exhibits a sharp rise in fluorescence when released into the extraliposomal fluid where the concentration is much lower. Consequently, antigen-antibody-mediated liposome lysis can be measured using CF released from the liposomes as an index.

In a previous study, we demonstrated that CRP antigen could be determined by the sandwich assay method using antibody-bearing liposomes and free antibody (secondary antibody) (5). Namely, CRP antigen dose-dependent liposome lysis was observed when liposomes bearing anti-CRP antibody (goat IgG fraction) were incubated with CRP antigen in the presence of secondary antibody (rabbit IgG fraction) and guinea pig complement. On the other hand, CRP antigen-dependent liposome lysis was hardly observed in the absence of secondary antibody because the binding of goat IgG to CRP antigen did not activate guinea pig complement effectively. When liposomes bearing rabbit IgG were employed in the assay, guinea pig sera as a source of complement induced CF release without the presence of CRP antigen. Hence, we used two sources of antibodies (goat for the attachment to liposomes and rabbit for secondary antibody) in the sandwich assay. However, the need for two antibody sources is a serious disadvantage, so additional efforts were made to improve the LILA system.

We investigated the application of mouse monoclonal antibodies to LILA to determine if it could induce antigen-

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dependent liposome lysis without requiring secondary antibody. We report herein the modified LILA using mouse monoclonal antibodies against CRP antigen, one of the acute-phase proteins.

Material and Methods

Lipids and other chemicals. Dipalmitoylphosphatidylethanolamine (DPPE) and cholesterol (Chol) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dipalmitoylphosphatidylcholine (DPPC) from Nippon Fine Chemical Co., Ltd. (Osaka, Japan). These lipids were used without further purification. DPPE with a dithiopyridyl group (DTP-DPPE) was used for coupling of antibodies to liposomes. DTP-DPPE was prepared according to the method of Leserman *et al.* (6). These lipids were dissolved in chloroform or chloroform/methanol (2:1), and stored at −20°C under nitrogen gas. Carboxyfluorescein (CF) was obtained from Eastman Kodak (Rochester, NY, USA) and purified as described by Weinstein *et al.* (7).

Purification of CRP (working standards). Pooled inflammatory human sera containing more than 50 μg/ml of CRP antigen were filtrated with a 0.45 μm Millipore filter (Bedford, MA, USA) and purified by affinity chromatography with agarose gel coupled to 2-aminoethanol dihydrogenphosphate ligand (8). CRP antigen prepared here showed a single protein band on electrophoresis using a 7.5% SDS-polyacrylamide gel. The CRP concentration was determined by single immunodiffusion test (N-Immunoring CRP, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). The stock CRP standard was stored at −75°C and a series of working standards was prepared by appropriately diluting the stock with gelatin veronal buffered saline (GVB^{-}; 145 mM NaCl, 3 mM 5,5-diethylbarbituric acid, 2 mM sodium 5, 5-diethylbarbiturate and 1 g gelatin per liter, pH 7.4) before use.

Mouse monoclonal antibodies. Hybrid cells producing antibodies to human CRP antigen were prepared by fusing myeloma cells (P3-X63-Ag8-U1) with spleen cells from BALB/c mice immunized with CRP antigen. Briefly, mice were injected intraperitoneally with an emulsion of purified CRP antigen (30 μg/mouse) and Freund's complete adjuvant (Difco Laboratories, Detroit, MI, USA), then boosted intravenously after 30 days with 10 μg of CRP antigen. The mice were killed 3 days later to obtain spleen cells for fusion (9). Hybridomas which specifically reacted with CRP antigen were screened by LILA using CRP antigen-bearing liposomes (4) and then cloned by limiting dilution (10).

Antibodies to CRP antigen were prepared from mouse ascitic fluid which was obtained by inoculation of hybridoma intraperitoneally into BALB/c mice primed with Pristane. The ascitic fluid was treated with 50% ammonium sulfate, and the antibody fraction was further purified on a column of DE-52 cellulose (Whatman, Maidstone, Kent, England) using 0.05 M Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl. The concentration of the IgG fraction was estimated from the absorbance at 280 nm. To determine the subclass of the monoclonal antibodies, the purified IgG fraction was concentrated and then examined by double-immunodiffusion using subclass-specific antisera.

Complement. Guinea pig sera (Shizuoka Agricultural Cooperative Association for Laboratory Animals, Hamamatsu, Japan) were used as a complement source. The sera were aliquoted and stored at −75°C until use. 1 U of complement (1 CH50) was defined as the amount of serum needed for 50% hemolysis of sensitized sheep red blood cells (5×10^8/7.5 ml) after incubation for 60 min at 37°C (11).

Modification of monoclonal antibody. Antibodies were modified with a heterobifunctional cross-linking reagent, N-hydroxysuccinimidyl-3- (2-pyridyldithio) propionate (SPDP) in the following manner (3): 2 mg/ml of monoclonal antibodies were incubated in 0.01 M Hepes-buffered saline (pH 7.4) containing 0.1 mM of SPDP for 30 min at room temperature. Unreacted SPDP was removed by passage through a Sephadex G-25 column equilibrated with 0.1 M acetate-buffered saline (pH 4.5), and IgG introduced DTP was reduced with 50 mM dithiothreitol (DTT) in 0.1 M acetate-buffered saline (pH 4.5) for 20 min at room temperature. The reaction mixture was passed through a Sephadex G-25 column equilibrated with 0.01 M Hepes-buffered saline to remove excess DTT. The IgG modified with SPDP and DTT was immediately used to prepare antibody-bearing liposomes.

Preparation of liposomes bearing anti-CRP antibody. CF-entrapped multilamellar liposomes were prepared from a lipid solution containing DPPC (0.5 μmol), Chol (0.5 μmol) and DTP-DPPE (0.02 μmol) by the method of Yasuda *et al.* (2). The antibody-bearing liposomes were prepared according to the method described previously (5). Briefly, a 500-μl

portion of freshly prepared liposome suspension (about 1 μmol of total lipid) was added to 500 μl of mouse IgG modified with SPDP and DTT at a concentration of 2.0 mg/ml, and then incubated overnight at room temperature with gentle shaking. Unbound IgG was removed by 20 min cycles of centrifugation (20,000 $\times$ g) in GVB^- at 4°C. After the final centrifugation, the pellet was resuspended in 1 ml of GVB^- and a portion of the suspension was analyzed for the organic phosphorus concentration (12). The amount of IgG on the liposomes was estimated from the difference in protein concentration in the supernatant before and after the coupling reaction using the method of Bradford (13).

Standard assay system. All dilutions were performed in GVB^{2+} (GVB^- containing 0.5 mM $MgCl_2$ and 0.15 mM $CaCl_2$). A 25-μl portion of serially diluted CRP antigen (working standards) and 5 μl of 100-fold diluted liposome suspension from stock liposomes were added to each well of a microplate (Nunc, Roskilde, Denmark), followed by the addition of 25 μl of appropriately diluted complement. After the mixture was incubated for 1 h at 37°C, 100 μl of veronal-buffered saline containing 10 mM disodium ethylenediaminetetraacetic acid (EDTA-VB; pH 7.4) were added to each well to terminate the reaction.

A microplate fluorometer, MTP-32 model (excitation: 490 nm, emission: 530 nm, Corona Electric Co., Katsuta, Japan) was used to measure the fluorescence of each well. Specific marker release (%) was calculated from the equation: $(F_1-A)\times 100/(F_2-A)$, where F_1 is the amount of CF released from the liposomes, F_2 is the total releasable fluorescence obtained by lysing with 25 μl of a 10% Triton X-100 solution instead of complement, and A is that obtained by adding 25 μl of GVB^{2+} instead of CRP antigen.

Enzyme immunoassay (EIA) using sandwich assay. A 96-well microplate (Nunc) was coated with monoclonal antibody (50 μg/ml of IgG fraction), and then blocked with 0.01 M phosphate-buffered saline (PBS; pH 7.2) containing 1% bovine serum albumin (BSA). Alkaline phosphatase (ALP)-labeled monoclonal antibodies were prepared by oxidation with sodium metaperiodate (14).

All dilutions were performed with PBS containing 0.1% BSA. A 100-μl portion of the CRP working standard was added to each well of the microplate in duplicate. After incubating for 1 h at 37°C, unbound CRP antigen was washed out with distilled water. Subsequently, a 100-μl portion of appropriately diluted ALP-labeled monoclonal antibody was added to each well. After incubation for an additional 1 h at 37°C, the microplate was again washed, and 100 μl of substrate (4.5 mM disodium phenylphosphate and 2 mM 4-aminoantipyrine in 25 mM carbonate buffer, pH 10) were added to each well. The enzyme reaction was terminated after 20 min by the addition of 50 μl of 0.8% sodium metaperiodate. The absorbance at 500 nm was read with an EIA analyzer (ETY-96, Oriental Inst., Ltd., Tokyo, Japan).

Results

To obtain antibodies having complement-activating capacity, hybrid cells were screened by LILA with liposomes bearing CRP antigen, and five clones were selected. Antibody secreted from each clone was prepared from mouse ascitic fluid, and used in the following investigation.

Determination of immunoglobulin subclass revealed the five antibodies (AC-1, -2, -3, -4, and -5) to be IgG1 (AC-1, -2, -3), IgG2b (AC-4), and IgG3 (AC-5), respectively. The reactivity of the purified antibodies (IgG fraction) to CRP antigen was tested by LILA. CF-entrapped liposomes composed of DPPC (0.5 μmol), Chol (0.5 μmol) and DTP-DPPE (0.02 μmol) were prepared. CRP antigen was then covalently coupled to the liposomes (about 100 μg per 1 μmol total lipid), which were then suspended in 1 ml of GVB^-, and were further diluted 100-fold with GVB^{2+} before use. Each antibody, adjusted to a concentration of 2 mg/ml, was diluted 1,000-fold and then serially diluted 2-fold for six dilutions with GVB^{2+}. A series of diluted antibody solutions was assayed according to the standard assay system.

Fig. 1 shows CF release curves obtained with the five monoclonal antibodies. The results indicate that the reactivity of AC-1 and -4 antibodies against liposome-bound CRP antigen was similar, and higher than that of AC-2, -3, and -5 antibodies in decreasing order.

Furthermore, an attempt was made to determine the difference in reactivity of each monoclonal antibody to CRP antigenic determinants using an EIA employing a sandwich method. Based on the assumption that the sensitivity of the EIA method decreases as the CRP antigenic determinants recognized with labeled antibody decreased (if both immobilized antibody and labeled antibody recognize the same CRP antigenic determinants), each of the monoclonal antibodies was separately immobil-

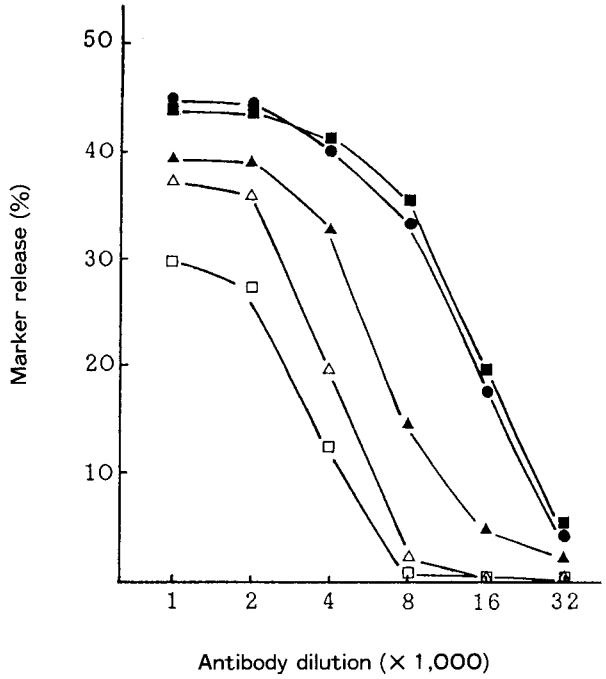
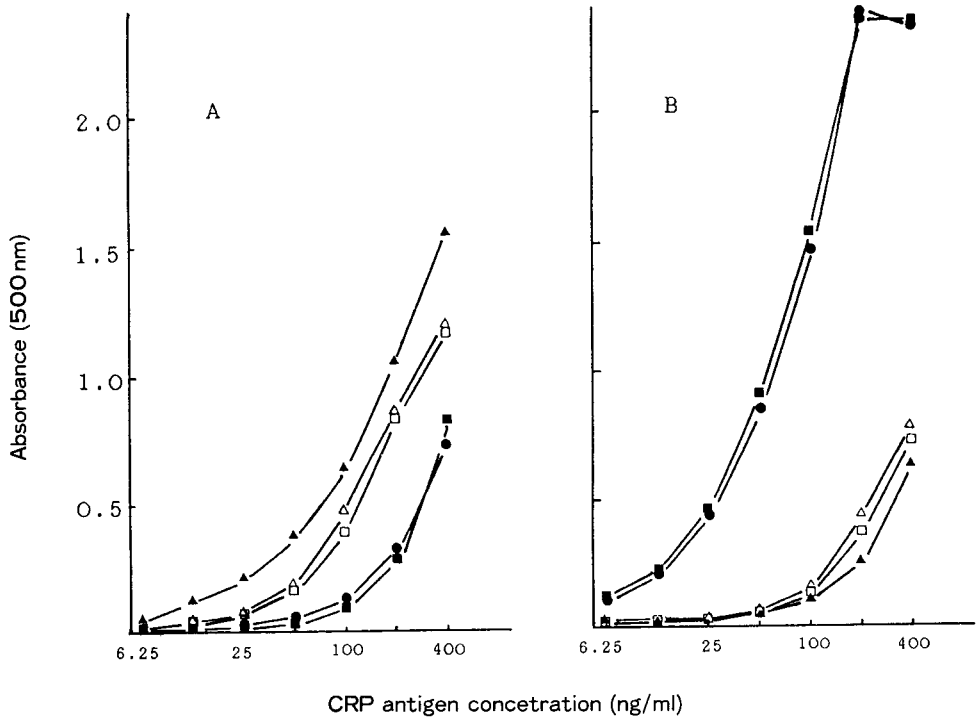


Fig. 1 (Upper) The reactivity of monoclonal antibodies against CRP antigen on liposomal membrane. Each monoclonal antibody, adjusted to 2 mg/ml, was serially diluted, and then assayed with liposomes bearing CRP antigen in the presence of complement (1 CH50). AC-1 (●), AC-2 (▲), AC-3 (△), AC-4 (■), AC-5 (□).

Fig. 2 (Lower) The difference in the reactivity of monoclonal antibodies against CRP antigenic determinants. Based on the assumption mentioned in the text, the difference in the reactivity of monoclonal antibodies was examined by enzyme immunoassay (EIA) using a sandwich assay with combinations of monoclonal antibody (AC-1 or AC-2) immobilized on a microplate and alkaline phosphatase (ALP)-labeled ones (AC-1 (●), AC-2 (▲), AC-3 (△), AC-4 (■), AC-5 (□)). Fig. A illustrates standard curves obtained by assaying under the combinations of AC-1 immobilized antibody and labeled ones. Fig. B illustrates standard curves obtained by assaying under the combinations of AC-2 immobilized antibody and labeled ones.



ized on a microplate, and labeled with ALP. EIA was performed using the immobilized antibody and each of the five ALP-labeled antibodies.

Fig. 2 shows the calibration curves obtained using EIA with combinations of AC-1 (or AC-2)-immobilized monoclonal antibody and the five ALP-labeled antibodies.

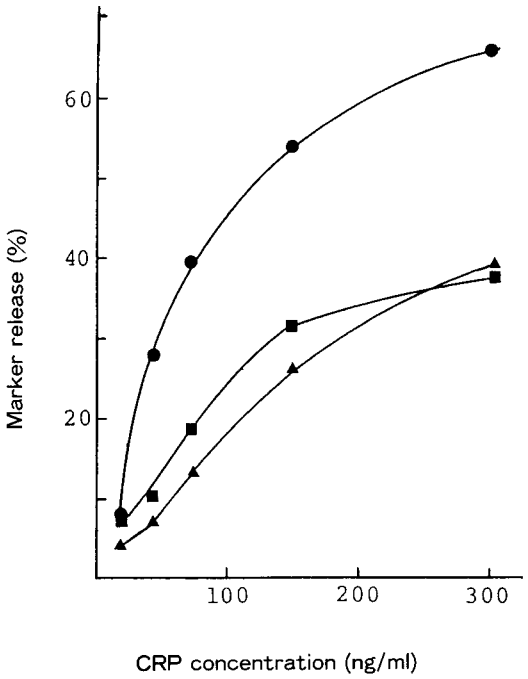


Fig. 3 Representative CF release curves obtained with liposomes bearing AC-1 and/or AC-2 monoclonal antibodies. A series of working standards was assayed in the presence of complement (4 CH50). The symbols used are as follows: X-bearing liposomes, X; AC-1 (▲), AC-2 (■), AC-1 and -2 (●).

As shown in Fig. 2A, the combination of AC-1-immobilized antibody and AC-1 (or AC-4)-labeled antibody gave lower sensitivity than other combinations. On the other hand, the combination of AC-2-immobilized antibody and AC-1 (or AC-4)-labeled antibody gave higher sensitivity than other combinations (Fig. 2B). These results indicate that the five monoclonal antibodies can be divided into two groups from the difference in reactivity to CRP antigen. One group is comprised of AC-1 and -4, and the other is made up of AC-2, -3 and -5. Antibodies in each group most likely recognize similar CRP antigenic determinants. The same results were also obtained from the combinations of AC-3, -4, or -5 antibody immobilized on a microplate and the five labeled antibodies (data not shown).

To covalently couple antibodies to liposomes, two monoclonal antibodies (AC-1 and AC-2) were chosen because they recognized different antigenic determinants and maintained the high reactivity, as shown in Figs. 1 and 2. We prepared liposomes bearing either AC-1 or AC-2 according to the procedure described in Materials and Methods. Similarly, liposomes bearing both antibodies (AC-1 and AC-2) were also prepared in the same manner except that the individual antibody solutions (2 mg/ml) were mixed at an appropriate ratio, and used for coupling. The amount of antibody bound to the liposomes was adjusted to 70 and 100 μg per 1 μmol total lipid, respectively.

Using the standard assay, we tested whether CF was released from liposomes bearing monoclonal antibodies (AC-1 and/or AC-2) without using a secondary antibody. The results shown in Fig. 3 demonstrate that CF was released in the absence of secondary antibody using liposomes bearing monoclonal antibody. Sensitivity was the highest when liposomes bearing the two antibodies were used in the assay. Furthermore, the sensitivity of this antibody combination was superior to the other combinations (data not shown). We also examined the most suitable ratio of AC-1 and AC-2, and the optimal CF release curve was obtained when a 1:1 ratio of the two antibodies was used for coupling (Fig. 3).

The effect of complement concentration on sensitivity was examined in the range of 1 to 5 CH50 since more than 5 CH50 resulted in the release of CF without CRP antigen. Although sensitivity increased with increasing complement concentration, the sensitivity obtained using liposomes bearing only one kind of antibody (AC-1 or

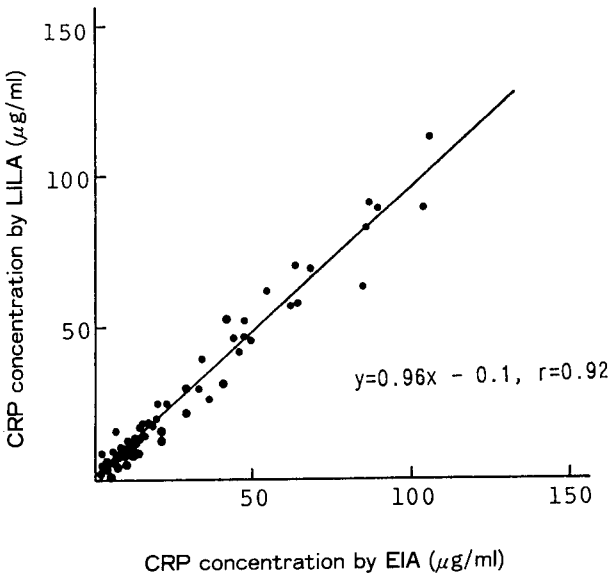


Fig. 4 Correlation study. The CRP concentrations in the sera of 100 patient were determined by enzyme immunoassay (EIA) and liposome immune lysis assay (LICA).

AC-2) was lower compared with both antibodies. An optimal CF release curve was obtained using liposomes bearing the two antibodies and 4 CH50 complement units, and covered a wide range of CRP concentrations between 5 and 300 ng/ml (Fig. 3).

The correlation between the LILA and EIA was examined by determining the sera of 100 patients. Patient sera were inactivated by heating for 30 min at 56°C, followed by adequate dilution (50-1200 times) in GVB^{2+} before measurements. Comparison of the result by linear regression analysis (Fig. 4) showed a high correlation between the LILA (y) and EIA (x) ($y = 0.96x-0.1$, $r = 0.92$).

Discussion

In this work, we demonstrated that use of monoclonal antibodies are advantageous in LILA system. The sandwich assay, described in our previous paper (5), required two different sources of antibodies (goat for the attachment to liposomes and rabbit as secondary antibody). This disadvantage was eliminated by using liposomes bearing monoclonal antibodies. In other words, antigen-dependent liposome lysis could be observed without the need for secondary antibody by using liposomes bearing two types of monoclonal antibodies even though the detectable concentration was nearly identical to that obtained using the sandwich assay and polyclonal antibody-bearing liposomes (data not shown). These results indicate that the choice of high-affinity monoclonal antibodies having complement-activating capacity as well as recognizing distinct antigenic determinants needed to improve the LILA system.

To obtain monoclonal antibodies suitable for this system, we screened hybridomas with LILA using liposomes bearing CRP antigen. This step seems to be important because, when monoclonal antibodies are used for LILA, not only their high affinity to CRP antigen but also their complement-activating capacity affects the sensitivity of the assay. With these considerations in mind, we obtained two monoclonal antibodies from clones selected by LILA.

Based on the results shown in Figs. 1 and 2, the two monoclonal antibodies were covalently coupled to liposomes. When these liposomes were used in assays, the highest sensitivity was obtained without secondary antibody (Fig. 3). This indicates that the combination of two or more monoclonal antibodies which recognize distinct antigenic determinants is effective for raising the sensitivity of the assay. This may be because, CRP antigen is more firmly fixed on liposomes using different monoclonal antibodies, so that effective complement activation is induced.

The use of monoclonal antibody in LILA appears useful for increasing the specificity and sensitivity of this assay system. We are convinced that LILA, using monoclonal antibody-bearing liposomes, will prove to be clinically a practical technique. Therefore, we are continuing our studies assaying other serum proteins with this system.

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