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A Novel Method for Screening Monoclonal Antibodies Reacting with Antigenic Determinants on Soluble Antigens; A Reversed Indirect-Enzyme Linked Immunosorbent Assay(RI-ELISA)

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ABSTRACT

A novel screening method was established to select new monoclonal antibodies which react with unknown antigenic determinants on molecules bearing antigen determinants reactive with established monoclonal antibodies. This new method is a sandwich assay termed "reversed indirect-enzyme linked immunosorbent assay" (RI-ELISA). Goat anti-mouse immunoglobulin antibodies are used as the primary immobilized antibody in this assay. They allow the non-purified monoclonal antibodies contained in hybridoma culture supernatants to bind to the microtest plate for enzyme immunoassay (EIA plate) much more efficiently than in the usual sandwich assay where the non-purified monoclonal antibodies are adsorbed directly to the polystyrene surface. The antigen solution is then reacted with the monoclonal antibodies and thereafter enzyme labeled monoclonal antibody with known specificity is added. Therefore, if the hybridoma culture supernatant contains monoclonal antibodies which were bound to the EIA plate and react with antigenic determinants on the soluble molecules which have antigen determinants recognized by the enzyme labeled antibody, the enzyme labeled antibodies will bind to induce an enzymatic reaction. The most important technical consideration in the RI-ELISA is the inhibition of direct binding of the enzyme labeled monoclonal antibodies to free sites remaining in the immobilized goat anti-mouse immunoglobulins antibodies. This problem could be effectively overcome by using normal mouse serum as blocking substance. These studies indicate that the RI-ELISA may be a useful screening method for selecting new monoclonal antibodies which react with unknown antigenic determinants on soluble molecules.

The monoclonal antibody technique has facilitated the detection of previously unknown tumor associated antigens (TAAs). Monoclonal antibodies against TAAs can be produced by immunizing with crude non-purified tumor extracts, tumor cells or tumor tissue, because the induction of monoclonal antibodies does not necessarily require purified immunogen. For the production of useful polyclonal antibodies, the antigen must be first purified to a degree and the resultant antibody must be absorbed to enhance specificity. But, on the contrary, purification of

antigen can be done after the production of monoclonal antibodies and the antibodies need no absorption to produce specificity.

Clinically useful soluble TAAs such as CA 19-9 reported by Koprowski et al[1,6], FH-6 by Fukushi et al[2,3], and KL-3 and KL-6 by us[5], are all tumor associated mucin antigens, which were recently discovered by use of the monoclonal antibody technique. However, the antibodies recognizing them were at first selected by screening on tumor and normal tissues and thereafter were demonstrated to be useful for detection of soluble TAAs. For the purpose of producing additional useful monoclonal antibodies which react with soluble TAAs, screening systems have to be developed in which specimens containing soluble TAAs are used as targets of uncloned monoclonal antibodies contained in hybridoma culture supernatants.

Tumor associated mucins have been shown to possess multiple heterogenous antigenic determinants on their molecules[7] similar to secreted blood group antigens. Such molecules may therefore possess additional unknown antigenic determinants which might serve as tumor markers. Thus we established a new screening method termed reversed indirect-enzyme linked immunosorbent assay (RI-ELISA) for detection of monoclonal antibodies reacting with unknown antigenic determinants on molecules having TAAs which can be recognized by known monoclonal antibodies. This report describes the usefulness of this RI-ELISA for such a screening method. Horseradish peroxidase labeled KL-3 (HRP-KL-3) antibody was used as a known enzyme labeled antibody and culture supernatant of KL-3 hybridoma as a model of a culture supernatant of a non-selected hybridoma. KL-3 antibody was chosen as it has been shown to detect TAA in pleural effusions derived from lung cancer by sandwich ELISA[5].

MATERIALS AND METHODS

Reversed indirect-enzyme linked immunosorbent assay (RI-ELISA) was performed according to the following procedures. Each well of 96-well microtest plate for enzyme immunoassay [EIA plate (Costar, Cambridge, Mass.)] was sensitized with 100 μl of 10 μg/ml goat anti-mouse immunoglobulin antibodies (Cappel Laboratories, Cochranville, Penn.) overnight at 4°C. After washing with TB-PBS [0.05% Tween 20, 0.1% BSA (bovine serum albumin), PBS (0.01 M phosphate, 0.14 M NaCl pH 7.4)], 100 μl of KL-3 hybridoma culture supernatant was added and allowed to react for one hr, after which the wells were washed. Thereafter, 100 μl of mouse serum buffer [10% normal mouse serum, 10% FCS (fetal calf serum), PBS] was added and allowed to react for one hr in order to block remaining anti-mouse immunoglobulin sites. After washing, an antigen solution diluted 10-fold with 10% FCS-PBS was added and allowed to react for one hr, after which the wells were washed, followed by addition of 100 μl of HRP-KL-3 antibody diluted 10-fold with 10% FCS-PBS and incubated for one hr. After washing, 100 μl of OPDA solution (0.3% *o*-phenylenediamine dihydrochloride, 0.02% H_2O_2, 0.15M citrate buffer pH 4.9) was added and allowed to react for 30 min, after which the reaction was stopped by adding 100 μl of 1N H_2SO_4 and absorbance (OD_{492}) was determined. A pooled pleural effusion derived from 20 pulmonary adenocarcinoma patients was used as the KL-3 antigen preparation and reagent diluents alone were used as negative controls in each corresponding reaction step.

RESULTS

The results of several types of ELISA are shown in Fig. 1. In the direct method in which antigen solution was directly bound to the EIA plate, no specific absorbance was observed as shown in Nos. 1 and 2. In the sandwich ELISA directly using culture supernatant of KL-3 hybridoma as the immobilized antibody, slight specific binding was observed as shown in Nos. 5 and 6. No effects of normal mouse serum on either of these two methods were seen as shown in Nos. 3, 4, 7 and 8. In the two types of RI-ELISA, as shown in Nos. 13, 14, 15 and 16, the reactions in the wells to which KL-3 antigen solution was added were considerably higher than those in the wells to which it was not added. Normal mouse serum strongly inhibited the binding of HRP-KL-3 antibody to goat anti-mouse immunoglobulin antibodies as shown in Nos. 9, 10, 11 and 12. By the addition of normal mouse serum, the reactions in the wells to which KL-3 antigen solution was not added and in those to which it was added decreased from 0.91 to 0.18

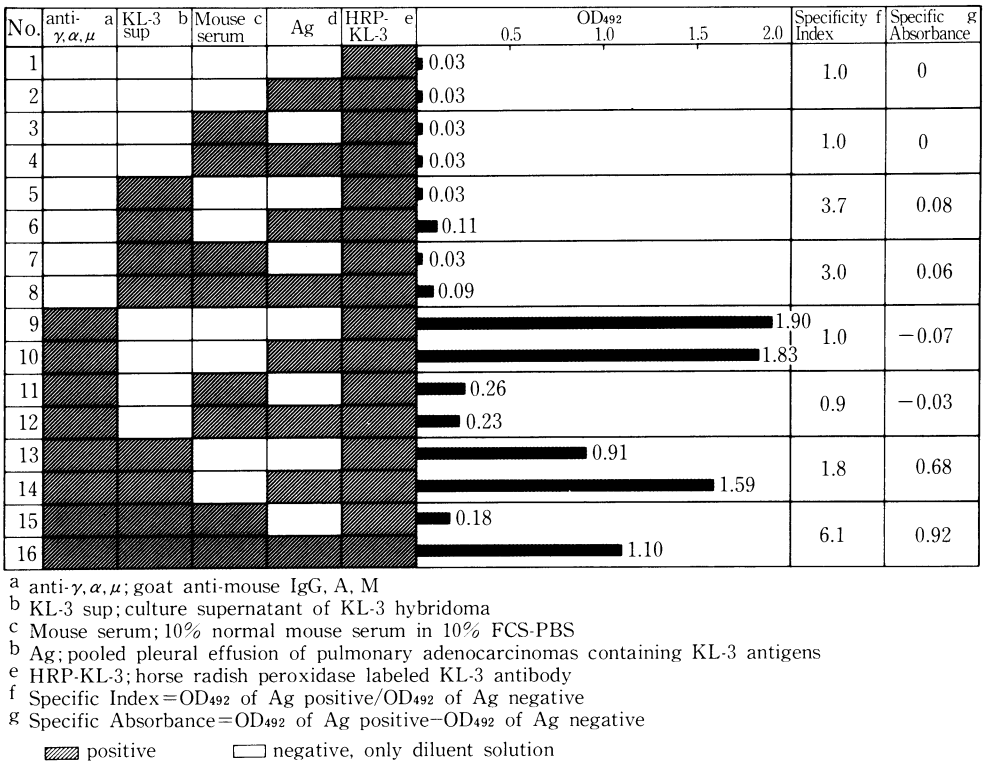

Fig. 1. Several types of ELISA were examined for their sensitivity by using KL-3 antibodies and a pooled pleural effusions as an antigen solution. In the direct method where we attempted to bind antigen directly to the plate (Nos. 1, 2, 3, and 4), no specific reaction developed. In a sandwich ELISA in which the culture supernatant of KL-3 hybridoma was used as an immobilized antibody (Nos. 5, 6, 7, and 8), a slight specific reaction developed. In RI-ELISAs, (Nos. 13, 14, 15, and 16), specific reactions developed with the most specific one developing in the case of where normal mouse serum was used as a blocking substance (Nos. 15, and 16). Normal mouse serum adequately inhibited the binding of horseradish peroxidase labeled KL-3 antibodies to the immobilized goat anti-mouse IgG, A, M antibodies (Nos. 9, 10, 11, and 12).

and from 1.59 to 1.10, respectively. However, the specificity was markedly improved with the specific index and specific absorbance increased from 1.8 to 6.1 and 0.68 to 0.92, respectively.

DISCUSSION

The technique of RI-ELISA and its superiority to the standard ELISA are described in this report. The specific absorbance on RI-ELISA was markedly higher than that in the standard ELISA in which the antibody containing hybridoma culture supernatant was used directly as the immobilized antibody. This high specific absorbance is due to the fact that most of the monoclonal antibodies in the hybridoma culture supernatant are linked to the goat anti-mouse immunoglobulin antibodies. Furthermore, the direct binding of horseradish peroxidase labeled antibodies to the remaining free goat anti-mouse immunoglobulin antibodies was inhibited by using normal mouse serum of high concentration. The mechanism of this inhibitive reaction is schematically shown in Fig. 2. These results suggest that RI-ELISA will be a useful screening method for detecting interesting monoclonal antibodies contained in unscreened hybridoma culture supernatants.

Another approach that has been used as a screening method is the binding inhibition assay in which soluble antigen is used as a target of non-selected monoclonal antibodies contained in hybridoma culture supernatants. Katz et al[4] have reported that some monoclonal antibodies capable of detecting soluble antigens were select-

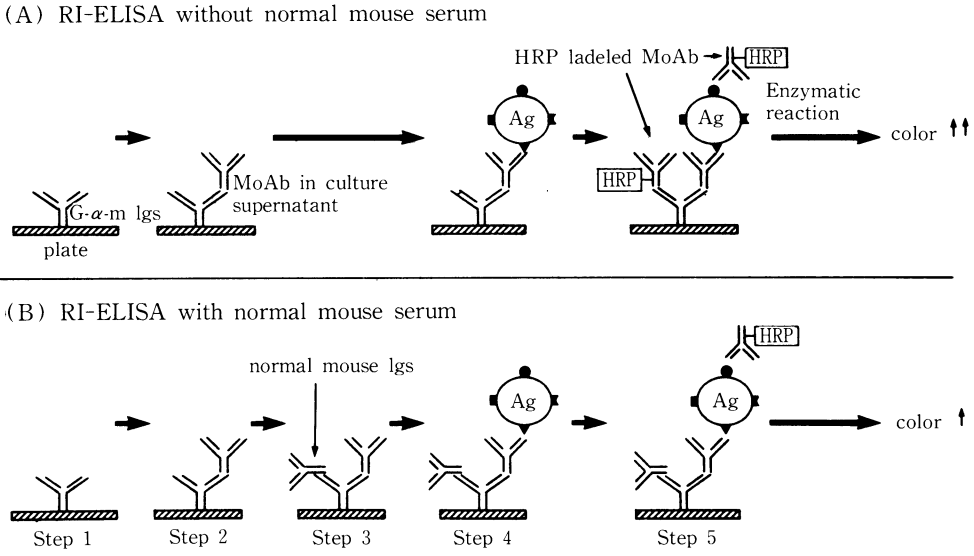

Fig. 2. In RI-ELISA not using normal mouse serum (A), horseradish peroxidase labeled monoclonal antibody (HRP labeled MoAb) may produce a background reaction by binding directly to goat anti-mouse IgG, A, M antibodies sites (G-α-mIgs) not filled by incubation with the test hybridoma culture supernatant. However, in RI-ELISA using normal mouse serum (B), mouse immunoglobulins (normal mouse Igs) in normal mouse serum used as a 3rd step fill the available sites and inhibit the direct binding of HRP labeled MoAbs to them, enhancing the specificity of the test.

ed by micro-enzyme-linked immunosorbent assay in which tumor cell extract was used as an immobilized antigen. We have also selected monoclonal antibodies by the binding inhibition assay in which a line of cancer cells was used as an immobilized antigen (data not shown). In the inhibition assay, soluble antigens competitively inhibit the binding of antibodies to the immobilized antigens. However, this assay has two major drawbacks. One is the difficulty of obtaining an adequate concentration of antibody in the hybridoma culture supernatant. There will be no inhibitory effect if an inadequate concentration is employed. The other drawback is due to the effect of other monoclonal antibodies contained in the same culture supernatant. Even if the binding of the desired monoclonal antibody were adequately inhibited by soluble antigens, the binding of other monoclonal antibodies contained in the culture supernatant would conceal the inhibited binding of the desired monoclonal antibody. This is because the majority of monoclonal antibodies in hybridoma culture supernatants must bind to their immunized extract or cells used as the immobilized antigen in the assay. Therefore, the inhibition assay can not be used as an efficient screening method for selecting monoclonal antibodies reacting with unknown soluble antigens.

Of course, unknown soluble antigen molecules cannot be detected by RI-ELISA, for only unknown antigenic determinants on known soluble antigen molecules will be selected. In describing this new screening method, this paper illustrates the model of RI-ELISA by using KL-3 antibodies which are known to detect soluble TAA in pleural effusion[5]. RI-ELISA may also be useful in selecting unknown antigenic determinants not only on TAAs but also on other kinds of soluble molecules.

We have begun selecting new monoclonal antibodies which detect soluble tumor associated mucins using the RI-ELISA and now are evaluating their clinical usefulness (data not shown).

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