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Short communication

A new purification method for the Fab and F(ab')$_2$ fragment of 145-2C11, hamster anti-mouse CD3ε antibody

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Recombinant protein G has been utilized in the purification of antibodies from various mammalian species based on the interaction of antibodies with protein G. The interaction between immunoglobulin and protein G may not be restricted to the Fc portion of antibodies, as many different F(ab')$_2$ or Fab fragments can also bind to protein G. I found both Fab and F(ab')$_2$ of 145-2C11, a hamster anti-mouse CD3ε antibody, bound to the protein G-sepharose. Interestingly, Fab and F(ab')$_2$ of 145-2C11 did not bind to the protein A-sepharose. The binding of Fab and F(ab')$_2$ of 145-2C11 to protein G provided a useful method to remove proteases, chopped fragments of the Fc region, and other contaminating proteins. The remaining intact antibody in the protease reaction mixture can be removed by using a protein A-sepharose, because the Fab and F(ab')$_2$ portions of 145-2C11 did not bind to protein A-sepharose. The specific binding of Fab and F(ab')$_2$ portions of 145-2C11 to a protein G-sepharose (though not to a protein A-sepharose) and binding of intact 145-2C11 to both protein A- and G-sepharose will be useful in developing an effective purification protocol for Fab and F(ab')$_2$ portions of 145-2C11.

Keywords: anti-murine CD3 ε antibody, Fab, F(ab')$_2$, protein A, protein G

Introduction

A hamster monoclonal antibody, 145-2C11, which is directed against CD3ε of the murine T cell receptor (TCR) complex is one of the most popular antibodies used in studies of immunology (Leo *et al.*, 1987; Tamura *et al.*, 1992). The 145-2C11 can activate murine T cells *in vivo* and *in vitro*. The activation and signal transduction mechanisms of TCR/CD3 complexes, stimulated by cross-linking with 145-2C11, have been studied for a decade. Most of the results were reached under the assumption that the cross-linking of TCR/CD3 complexes by 145-2C11 could mimic the real situation, where TCR/CD3 on the T cells bind MHC:Ag on antigen presenting cells (APC). This assumption may be untrue if intact 145-2C11 were used *in vivo*. Cross-linking of the CD3/TCR complex by intact 145-2C11 induces T cell proliferation, IL-2 receptor expression, and lymphokine production. However, injection of the F(ab')$_2$ fragments of 145-2C11 into mice results in the modulation of the TCR complex, but does not induce lymphokine secretion, IL-2 receptor expression or proliferation (Hirsch *et al.*, 1988; 1989; 1991). The reason for the different activities of F(ab')$_2$ versus intact 145-2C11 is unclear. The Fc portion of the antibody may be necessary for efficient multi-valent crosslinking between T cells and other FcR bearing cells, such as macrophages and B cells.

The decade-old findings may be very important in studies of costimulation of T cells. The signals induced from the TCR/CD3 complex and other costimulatory molecules, including CD28, CTLA4, and 4-1BB, could be modified if FcγR-expressing APC were present in the system. Intact 145-2C11 antibodies used for ligation of TCR/CD3 complexes may enhance, in the duration and/or the strength, the binding of FcγR-expressing APC to T cells. Forced cell-cell interactions between T cells and FcγR-expressing APC could induce other activation signals through unidentified costimulatory molecules. To ligate the TCR without other interactions between T cells and APC, usage of F(ab')$_2$ of 145-2C11, or F(ab) followed by F(ab')$_2$ of anti-hamster IgG, were necessary.

In this study I report findings that are useful for preparation of F(ab')$_2$ of 145-2C11. I found that F(ab) and F(ab')$_2$ of 145-2C11 bind to a protein G-sepharose column, but not to a protein A-sepharose column. However, intact 145-2C11 bound to both a protein G-sepharose column and a protein-A sepharose column. These characteristics of 145-2C11, a anti-mouse CD3ε antibody, provide the basis for a useful method to prepare the F(ab) and F(ab')$_2$ fragments of 145-2C11.

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Materials and Methods

Antibodies and reagents The anti-CD3ε hamster IgG (145-2C11) was obtained from the American Type Culture Collection (ATCC CRL-1975, Rockville, USA). Biotin-conjugated F(ab')$_2$ fragment of goat anti-hamster IgG (H+L) and Fc fragment specific FITC conjugated F(ab')$_2$ of rabbit anti-mouse IgG were purchased from Jackson Immunoresearch Laboratories, Inc., (West Grove, USA). Protein G-sepharose and protein A-sepharose were purchased from Sigma Chemical Co., (St. Louis, USA).

Production of anti- CD3ε antibody, 145-2C11 The 145-2C11 hybridoma was maintained in a complete medium containing RPMI1640 (Sigma Chemical Co., St. Louis, USA) supplemented with heat-inactivated 10% bovine calf serum (BCS) (Logan, USA), glutamine (2 mM), nonessential amino acids (0.1 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), pyruvate (0.1 mM), HEPES (25 mM), and β-mercaptoethanol (5.5×10^{-5} M) at 37°C, 5% CO_2. The cell line was checked regularly for the presence of mycoplasma and was mycoplasma negative.

The hybridoma cells were cultured in a complete media to prepare the initial cell mass. Cells were then harvested by centrifugation and resuspended in a HB101 serum free media (Irvine Scientific, Santa Ana, USA) at 1×10^6 cells/ml. After a 10 day-incubation in the 5% CO_2 incubator, the cell culture supernatant was harvested by high-speed centrifugation at 1000× g for 20 min. The clear supernatant was then filtered through a 0.4 mm filter. The resulting supernatant was ultrafiltrated by 1/10 (v/v), and precipitated by saturated ammonium sulfate (SAS) (50% final concentration of ammonium sulfate). The supernatant was incubated overnight at 4°C with gentle stirring. The precipitated antibody was collected by high-speed centrifugation (6,000×g, 45 min). The precipitate was resuspended with a minimal volume of PBS buffer (pH 7.2) and dialyzed against a PBS buffer (pH 7.2) for 2 days with multiple changes of the buffer.

Binding of intact 145-2C11 to both protein A- and protein G-sepharose columns Using a protein G- or a protein A-sepharose column to remove other serum proteins purified the 145-2C11 antibody. The protein G-sepharose or protein A-sepharose was packed in a mini column and washed with 20 mM phosphate buffer (pH 7.2) until the OD_{280} was at the base line. The SAS precipitated and dialyzed antibody solution was loaded on the gel several times and washed with 10 ml of 20 mM phosphate buffer (pH 7.2). The bound antibodies were eluted with elution buffer (0.1 M glycine-HCl, pH 2.7), and collected drop-wise into glass tubes containing pH 9.0, 1 M Tris buffer. The pH of the eluted sample was adjusted to 7.2 with 1 M Tris buffer (pH 9.0). The antibody fraction was pooled and dialyzed against PBS (pH 7.2). The dialyzed antibody was concentrated by ultrafiltration (MW cut off 30,000) to a final concentration of 2 mg/ml.

Pepsin digestion of 145-2C11 The antibody 145-2C11 was dialyzed against a 0.2 M acetate buffer (pH 2.8) overnight. One mg of lyophilized pepsin-agarose powder (3,200-4,500 units) was suspended in 1 ml of deionized distilled water and added to 1 mg of antibody. The antibody and pepsin reaction mixture was incubated at 37°C for 4 h, after which 2 M Tris was added to the mixture to stop the enzyme reaction and to bring the pH to 7.0. The reaction mixture was centrifuged at 1000×g for 10 min at 4°C in order to remove the pepsin-agarose. The supernatant was dialyzed against PBS (pH 7.0) overnight.

Binding test of Fab and F(ab')$_2$ fragments of 145-2C11 to protein A- and protein G-sepharose The pepsin digested samples of 145-2C11, containing a mixture of the intact antibody, and the F(ab) and F(ab')$_2$ fragments of 145-2C11, were used to test the binding ability to protein A-sepharose column and protein G-sepharose column. The pepsin-digested and dialyzed supernatant was loaded on the protein A-sepharose column or the protein G-sepharose column, and bound proteins were eluted by the same methods as mentioned above. Each step was monitored by optical density (280 nm) and the final F(ab) and F(ab')$_2$ fragments of 145-2C11 were analyzed by SDS-PAGE under reducing and nonreducing conditions.

Flow Cytometric Analysis Cells (5×10^5 cells/50 μl) were incubated for 30 min with the intact antibody, or a mixture of F(ab) and F(ab')$_2$ fragments of 145-2C11 on ice. Cells were washed three times with HBSS containing 2.5% BCS and 0.1% sodium azide (HBSS/BCS/AZ). Cells were further incubated for 30 min with FITC-conjugated goat anti-hamster IgG (H+L specific) antibody, or FITC-conjugated goat anti-mouse IgG (Fc specific) antibody on ice. Cells were washed 3 times, resuspended in 0.2 ml of HBSS/BCS/AZ, and analyzed using a FACSCalibur (Becton Dickinson & Co., San Jose, USA).

$[Ca^{2+}]i$ measurements For intracellular calcium studies, cultured 2E7 cells were resuspended to 1×10^7 cells/ml in RPMI 1640 containing 0.1% BSA and 3 μM fura-2-AM. After incubation at room temperature for 15 to 20 min, cells were diluted 5-fold with RPMI 1640 containing 0.1% BSA, and then incubated at 37°C for an additional 15 to 20 min. After washing with RPMI 1640 containing 0.1% BSA, the cells were pelleted, resuspended in HBSS with 0.1% BSA (HBSS/BSA) to 1×10^7 cells/ml, and kept on ice until use.

The cells were incubated on ice with the intact 145-2C11 antibody, or a mixture of F(ab) and F(ab')$_2$ fragments of 145-2C11 for 25 min, and washed once with HBSS/BSA. The bound antibodies were cross-linked with 50 μg of F(ab')$_2$ fragments of goat anti-hamster IgG (H+L specific) in the cuvette. The fluorescence of cell suspensions that were kept at 35°C with constant stirring was continuously monitored with a spectrofluorometer (SPEX Industries, Inc., Edison, USA) using 340 and 380 nm excitation wavelengths and a 510 nm emission wavelength. Calcium concentrations were calculated as described by Grynkiewicz *et al.* (1985).

Results and discussion

Protein G is a bacterial cell wall protein isolated from group G streptococci. Cloning and DNA sequencing analysis of protein G show three binding domains for immunoglobulin, albumin, and cell surface molecules (Sjobring *et al.*, 1988). The

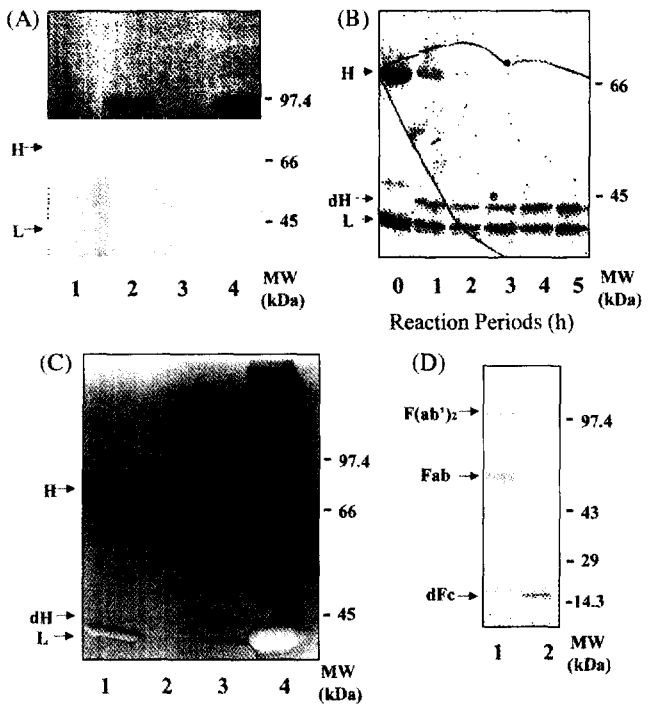

Fig. 1. SDS-PAGE analysis of eluents from protein A, or protein G column. A. Intact 145-2C11 was loaded on a protein A (lane 1 and 2), or a protein G column (lane 3 and 4). Bound (lane 1 and 3) and unbound fractions (lane 2 and 4) were collected and analyzed. Only the bound fraction contained a heavy (H) as well as a light chain (L) of 145-2C11. B. Purified intact 145-2C11 was digested with pepsin for various reaction periods. The reaction mixture was analyzed by SDS-PAGE under reducing conditions. The bands for the digested heavy chain (dH) appeared from 1 h and increased their density up to 4 h during incubation with pepsin. C. Pepsin-digested 145-2C11 was loaded on the protein A (lane 2), or protein G columns (lane 3) and the bound fraction was analyzed by SDS-PAGE under reducing conditions. The intact purified 145-2C11 (lane 1) and partially purified by SAS (lane 4) were also analyzed. D. Bound fraction (lane 1) and unbound fraction (lane 2) from the protein G column were analyzed under non-reducing conditions. The Fc portion of the heavy chain was digested by pepsin and the largest digested fragment of Fc portion (dFc) was 16kDa.

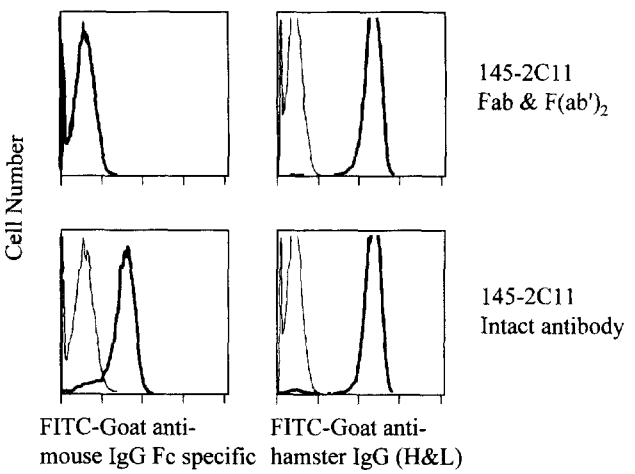

Fig. 2. Binding of intact antibodies (lower row) and Fab and $F(ab')_2$ fragments (upper row) to 2E7 $CD3\varepsilon^+$, $Fc\gamma RII/III^-$ T cell hybridoma. In the left column FITC-labeled goat anti mouse IgG (Fc specific) antibody was used, and in the right column FITC-labeled goat anti-hamster IgG (H+L specific) antibody was used.

recombinant protein G, in which the binding domains for albumin and cell surface molecules are removed by gene manipulation, is commercially available. The recombinant protein G has been thought to bind to immunoglobulin through the Fc portion of the antibody. However, the binding domain of immunoglobulin may not be restricted to the Fc portion of immunoglobulin, since some F(ab) or $F(ab')_2$ fragments also bind to the protein G molecules (Erntell *et al.*, 1988; Proudfoot *et al.*, 1992; Derrick *et al.*, 1994). The binding may be due to structural similarities between the F(ab) domain of certain antibodies and the Fc region.

The antibody binding ability of the protein A column and the protein G column was confirmed by their ability to bind intact 145-2C11. The Hybridoma 145-2C11 culture broth was concentrated using a 50% ammonium sulfate. The dialyzed 145-2C11 solution was loaded onto a protein G-sepharose column and a protein A-sepharose column. Eluents were collected and pooled. The fractions were collected based on their optical densities and analyzed by SDS-PAGE under reducing conditions (Fig. 1-A). The eluted fraction from both columns contained pure intact 145-2C11. These data proved the binding ability of the protein A and the protein G columns to the intact form of the 145-2C11 antibodies.

The pepsin-agarose was used for the digestion of 145-2C11 to produce $F(ab')_2$ with an easy removal of enzyme activity by centrifugation. The digestion condition was determined by optimizing the enzyme digestion period under fixed concentrations of antibody and enzyme, the pH of reaction buffer, and the reaction temperature. Complete digestion of intact 145-2C11 into $F(ab')_2$ was noticed after a 3 h incubation (Fig. 1-B), therefore a 3.5 h time frame was chosen for the digestion period in further experiments.

The pepsin-digested 145-2C11 was loaded onto a protein G column, or a protein A column. The eluent was collected and analyzed by SDS-PAGE under reducing (Fig. 1-C), or non-reducing conditions (Fig. 1-D). The pepsin reaction mixture contained 110kD $F(ab')_2$, 50-55kD F(ab), and 16kD digested fragments of the Fc portion of heavy chain under non-reducing conditions. Interestingly, the protein G-sepharose retained both F(ab) and $F(ab')_2$ fragments of 145-2C11, but not the 16 kD fragment. Although we do not know whether protein G binds to the F(ab) portion, or to the remaining fraction of the Fc portion of the heavy chain, we do know that the binding of both the F(ab) and $F(ab')_2$ fragments of 145-2C11 to protein G-sepharose was reproducible. However, neither F(ab) nor $F(ab')_2$ fragments of 145-2C11 were detected

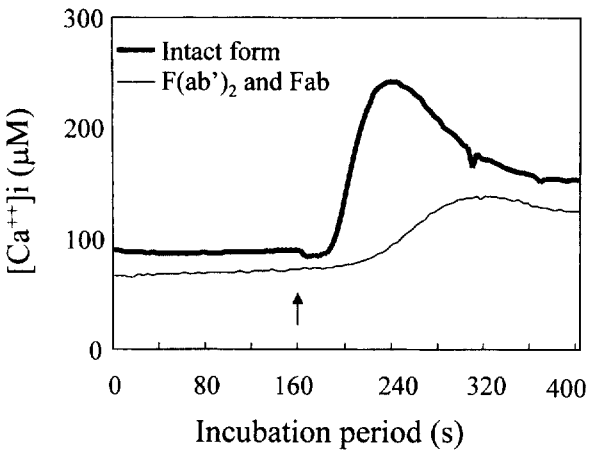

Fig. 3. Internal calcium concentrations of 2E7 cells after cross-linking CD3e. The 2E7 cells were incubated with intact (solid line), or Fab and F(ab')$_2$ fragment mixture (thin line) of 145-2C11 on ice, and cross-linked with goat anti-hamster IgG (H+L) in the cuvette (arrow).

in the eluent of the protein A- sepharose column (Fig. 1-C).

The F(ab) and F(ab')$_2$ fragments of 145-2C11 were tested for their ability to bind to CD3ε on T cells. The murine γdT cell hybridoma, 2E7 (which does not express any FcγR) was used to test the binding of the fragments. A goat anti-mouse IgG antibody, which is specific for Fc and has crossreactivity to hamster IgG, was used as a secondary antibody. This secondary antibody could bind to 2E7 cells that are coated with the intact form of 145-2C11, but not to the cells that are coated with F(ab) and F(ab')$_2$ fragments of 145-2C11. In contrast, a goat anti-hamster IgG (H+L) specific antibody could bind to cells that are coated with either intact or F(ab) and F(ab')$_2$ fragments of 145-2C11 (Fig. 2). These findings suggest that the binding ability of F(ab) and F(ab')$_2$ fragments of 145-2C11 are similar to that of the intact antibody.

The signal inducing ability of F(ab) and F(ab')$_2$ fragments of 145-2C11 was also tested for their functional activity. The induction of an internal calcium concentration was determined by cross-linking CD3 using intact 145-2C11, or a mixture of F(ab) and F(ab')$_2$ fragments. As shown in Fig. 3, cross-linking surface CD3 molecules with intact 145-2C11, or F(ab) and F(ab')$_2$ fragments of 145-2C11, increased the internal calcium concentrations. Interestingly, the peak of the internal calcium concentration induced by the cross-linking of surface CD3 molecules with F(ab) and F(ab')$_2$ fragments of 145-2C11 followed by a secondary antibody was lower than that by intact 145-2C11 followed by a secondary antibody. The efficient cross-linking of CD3 may be important in the early internal calcium movement that forms the calcium influx peak. The anti-hamster (H+L) antibody may bind and cross-link efficiently to intact 145-2C11 more than to Fc-less 145-2C11. However, the internal calcium concentrations in the cells had similar 240s after cross-linking CD3 with both treatments. These results suggest that cross-linking the F(ab) and F(ab')$_2$ fragments, followed by the anti-hamster antibody, may be less efficient in the early stages, but induce a similar strength of signals in the whole process, as compared to cross-linking the intact 145-2C11 followed by the anti-hamster antibody.

The cross-linking of intact 145-2C11 by the anti-hamster (H+L) antibody may be more efficient than that of the Fc-less antibody. This situation may be important if a very short reaction time is allowed. This difference, however, may be covered if enough reaction time, and a saturated amount of anti-hamster (H+L) antibody, is allowed. Enough incubation time, and a saturated amount of secondary antibody for the binding to intact or Fc-less 145-2C11, was allowed in the FACS staining conditions. A similar amount of the FITC-conjugated anti-hamster antibody, bound to intact and Fc-less 145-2C11, attached to the cell surface (Fig. 2).

From these observations, a simple purification method for the F(ab) and F(ab')$_2$ fragments of 145-2C11 could be developed. The pepsin-digested 145-2C11 can be purified using protein G column in order to remove contaminating proteases and fragments of the Fc portion. The eluents from the protein G column, which contain the intact antibody (if pepsin-digestion was not complete) and the F(ab) and F(ab')$_2$ fragments of 145-2C11, are passed through the protein A column. The non-bound fraction will be a mixture of F(ab) and F(ab')$_2$ fragments of 145-2C11. Therefore, contaminating proteases, fragments of the Fc portion, and intact 145-2C11 will be removed from the pepsin-digested mixture by consecutive passages through the protein G-sepharose column and the protein A-sepharose column.

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