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IMMUNOAFFINITY ISOLATION OF THE LEWIS BLOOD GROUP ANTIGENS

SUSAN C. FRANTZ*, HORACE F. HENRIQUES and TERRY M. PHILLIPS
Immunochemistry Laboratory, Room 413 Ross Hall, The George Washington University Medical Center, 2300 Eye Street, N.W., Washington, DC 20037 (U.S.A.)

SUMMARY

Extraction, followed by immunoaffinity chromatography on a column of immobilized antibodies is described for the isolation of the Lewis blood group antigens. These affinity-isolated antigens are suitable in organ transplant immunology laboratories for screening potential donors and recipients for mismatch at the Lewis antigen loci.

INTRODUCTION

Prior to 1978, Lewis antigens were not considered significant in matching recipient and donor for kidney transplantation. Since that time, research has provided evidence that the Lewis antigens may cause sensitization, resulting in graft rejection. New techniques, more sensitive than the standard red cell agglutination, have permitted the detection of low levels of anti-Lewis antibodies in Lewis-negative patients, transplanted with Lewis-positive kidneys. In all of these cases, graft rejection episides had occurred[1]. Cytoxicity testing, performed by Oriol *et al.*[2], indicated the potential of Lewis antigens to serve as "targets for antibody-mediated lymphocyte lysis".

Graft rejection could occur when the Lewis antibody is present, because the Lewis antigen is found in epithelial cells of the distal convoluted and collecting tubules of the kidney[3]. The potential role of the Lewis antigen in eliciting rejection is still unclear, and the significance of this antigen group is under debate[4,5]. One possible reason for the lack of testing for the presence of antibodies directed against this antigen group is the difficulty with which the antigen itself is isolated and purified for use in sensitive screening assays.

In an effort to introduce sensitive anti-Lewis antibody screening in our transplant center, we have developed a technique for isolating Lewis antigens from red blood cell membranes. The combination of extraction and immunoaffinity chromatography with commercially available anti-Lewis typing antibodies is described below.

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EXPERIMENTAL

Materials

Blood group O negative human red blood cells, carrying the Lewis^a antigen, were purchased from American Dade (Miami, FL, U.S.A.). Commercially available goat anti-human Lewis^a and Lewis^b antiserum and red blood cell typing antisera against human blood groups, A. B, K, E, M, N, S, s, C, c, E, e, D, Fy^a, Jk^a, and Jk^b were purchased from Ortho Diagnostics (Raritan, NJ, U.S.A.). Glycophase controlled-pore glass beads (pore size 200 Å) which have reactive N-hydroxysuccinimide ester side-chains bonded to the bead surface were obtained from Pierce (Rockford, IL, U.S..A.). Sephadex G-200 packing was obtained from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.) and allowed to swell in 0.01 *M* phosphate-buffered saline (PBS), (pH 7.0) prior to use. Radial immunodiffusion plates for estimating IgM levels were obtained from Kallstad (Austin, TX, U.S.A.). Agarose and all laboratory chemicals were purchased from Sigma (St. Louis, MO, U.S.A.). The chromatography columns and fittings, and the dot blot apparatus and nitrocellulose membranes were obtained from Biorad Labs. (Rockville Centre, NY, U.S.A.).

Lewis antigen extraction

A 2-ml volume of a 10% suspension of Lewis^a-positive, O-negative red blood cells were washed three times in 10 ml of PBS (pH 7.3) and a 2-ml suspension of 3–5% cells was lysed by adding an equal volume of toluene to the cell suspension, and mixing for 4 min and then allowed to separate overnight at room temperature[6]. The lysate was centrifuged at 1500 *g* for 5 min before the aqueous layer was carefully removed and discarded. The organic layer was retained for further isolation by immunoaffinity chromatography. The red cell membrane pellet was re-extracted by repeating the procedure described above, and the second organic layer was pooled with the organic layer obtained from the first extraction.

Size-exclusion isolation of the IgM anti-Lewis^a antibody

The goat IgM anti-Lewis^a antibodies were isolated from the whole serum by size-exclusion chromatography on Sephadex G-200. The G-200 gel was swollen in PBS for 72 h at room temperature and then degassed under vacuum. The gel was gravity packed into a 20 × 1 cm glass column, which had been connected to a peristaltic pump and equilibrated for 6 h with PBS, at a flow-rate of 0.8 ml/min. The goat serum (2 ml) was applied to the column and chromatographed at a flow-rate of 0.8 ml/min, using PBS as the eluent buffer; 1-ml fractions were collected in a ISCO (Lincoln, NB, U.S.A.) fraction collector. The fractions were assayed for the presence of the anti-Lewis^a antibody by the standard red cell agglutination technique described by the manufacturer, using the commercial Lewis^a antigen-positive human red blood cells[7]. The agglutination-positive, antibody-containing fractions were pooled (Fig. 1), and the pH was adjusted to 7 prior to coupling with the controlled-pore glass beads. The IgM content was measured by radial immunodiffusion and contamination with other serum components checked by immunoelectrophoresis.

Preparation of immunoaffinity column

The glass beads were throughly washed with distilled water prior to coupling

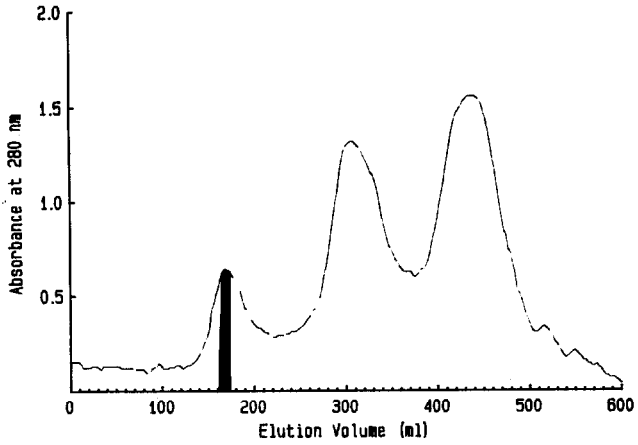

Fig. 1. Chromatogram of the Sephadex G-200 isolation of the goat anti-human IgM antibody. The shaded area under the first peak was the material used as the immobilized antibody. Running conditions are described in the text.

the goat anti-Lewis^a antibody with the reactive side-chains on the bead surface. Then, 350 μg of the antibody was added to 2 g of the N-succinimide ester-derivatized glass beads. The mixture was adjusted to pH 7.9 by the addition of 0.01 *M* phosphate buffer and then placed on a rocker overnight at room temperature to allow the anti-Lewis^a antibody to bind to the surface of the glass beads[8]. The antibody-coated beads were gravity-packed into a 10 × 0.5 cm column and equilibrated with PBS.

Immunoaffinity isolation of the Lewis^a antigen

The immunoaffinity column was stabilized in methanol–PBS (3:1) by passing 20 ml of this mixture through the column at 1 ml/min. The Lewis antigen extract (2 ml) was applied to the column and allowed to be absorbed by the column before the unbound material was eluted with 50-ml of the eluent mixture. The Lewis antigen was eluted by using a steep acid elution gradient, generated by adding 5 ml of 1 *M* acetic acid[9] to the running buffer and maintaining the upper limit of the gradient for a further 40 min. The elution profile was monitored at 280 nm and 500-μl fractions were collected throughout the run. These fractions were pooled and concentrated by ultrafiltration (Amicon, Danvers, MA, U.S.A.). The ten-fold concentrated immunoaffinity fraction was tested for the presence of the Lewis^a antigen by tube flocculation, using the original anti-Lewis^a antiserum.

Immunodiffusion studies

To determine specificity, the concentrated eluate was assayed by agarose double diffusion[10] for the presence of both Lewis^a and Lewis^b antigens. In addition, the immunoaffinity isolate, was tested for the presence of other blood group antigens (A, B, K, E, M, N, S, s, C, c, E, e, D, Fy^a, Fy^b, Jk^a, and Jk^b that may be present. Agarose (1g) (Sigma) was suspended in 100 ml of PBS and gentle boiled until the solution became clear. At this point, the solution was boiled for another 2 min, and the molten agarose was poured on glass plates to a thickness of 2 mm. When the

agarose had solidified, a central well, surrounded by four, equidistant peripheral wells was cut from the agarose gel, and 20 μl of the immunoaffinity isolated Lewis^a antigen was placed in the center well of five individual gels. A 20-μl volume of each of the commercial blood group antisera was placed in different peripheral wells, and the gels were incubated in a most atmosphere at room temperature for 24 h. Then the gels were examined and the presence of visible precipitin arcs was recorded.

Dot blot analysis

As a specificity check of the immunoaffinity-isolated Lewis^a antigen, dot blot analysis was performed, using nitrocellulose paper and a Biorad dot blot apparatus. The nitrocellulose paper was allowed to swell in 0.01 M phosphate buffer and placed into the apparatus by the technique described in the instruction manual[11]. A 100-μl volume of the immunoaffinity-isolated antigen was placed into each well and allowed to bind to the membrane for 24 h at room temperature. Aliquots (100 μl) of the typing antisera were added and incubated with the immobilized antigen for 2 h at room temperature. The membrane was washed in PBS by vacuum, and the bound antibodies were detected by using gold-labelled Protein A[12].

RESULTS AND DISCUSSION

A simple technique is described for the isolation of Lewis blood group antigens by lysis of human red blood cells in organic solvent, followed by recovery of the organic phase by centrifugation and application to an immunoaffinity column.

Although the membrane extraction was simple, difficulty was encountered in immobilizing the G-200 purified goat IgM anti-Lewis antibody on a suitable column packing medium. Functional amino groups, suitable for immobilization appeared to be present on the antigen receptors of the molecule. Coupling via these groups would result in the loss of a functional arm of the antibody. However, as IgM is large (mol.wt. 900 000) and contains five antibody units per intact molecule, it was decided to couple this antibody with controlled-pore glass beads via a N-hydroxsuccinimide ester and lose one of the antibody units. This procedure is easy to perform, and the beads were found to bind 270 μg of the IgM antibody per g of derivatized beads. This measurement was calculated after examining the post-immobilization eluate for the presence of IgM by radial immunodiffusion. Immunoelectrophoretic analysis of the IgM demonstrated the presence of approximately 4% α-2-macroglobulin, and trace amounts of serum lipoproteins.

Isolation of the Lewis^a antigen was performed on a short, wide-bore column. The immunoaffinity-retained antigen was recovered by elution in acid medium (Fig. 2). The flocculation studies performed on the eluate from the immunoaffinity column demonstrated that detectable levels of Lewis^a antigen could be extracted from human red blood cells by this technique. The recovered material was found to contain only the Lewis^a antigen when tested by agarose gel immunodiffusion against a battery of antisera, directed against the other common blood group antigens, including Lewis^b antigen.

The specificity of the immunoaffinity-isolated Lewis^a antigen was confirmed by the sensitive dot blot assay. Of particular interest was the finding that even at this high sensitivity, no cross-contamination with other factors could be detected.

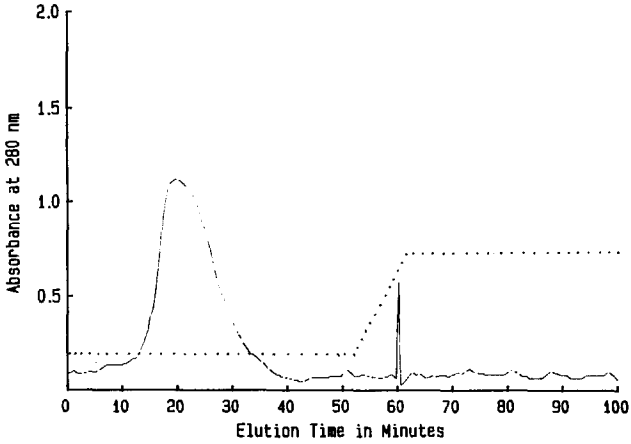

Fig. 2. Chromatogram of the immunoaffinity isolated Lewis antigen. The primary peak in the unbound membrane material and the second peak is the affinity isolated antigen. The dotted line represents the acid gradient. Running conditions are described in the text.

The Lewis antigens are high-molecular-weight glycosphingolipids, which share structural similarities with the major A, B and H antigens of the ABO system[13]. It has been shown that the Lewis antigens and the A, B and H antigens are derived from the same precursor molecule and that the expression of the Lewis antigen is under the same genetic influence as is the expression of the major blood group antigens[14]. In light of this, the necessity of cross-matching potential organ donors and recipients for Lewis antigen becomes more important than has previously been believed[15,16]. One of the controversial aspects surrounding this group of antigens is that they apparently cannot be detected by conventional techniques[7]. However, other investigators have shown that newer, more sensitive immunological assays can be used to detect low levels of these antibodies, which appear to be increased following rejection episodes[17].

Another aspect which makes this group of antigens significant in the kidney transplantation field is an indication that the Lewis antigens are actively secreted by the tubular epithelial cells[3]. In situations where a Lewis antigen-positive organ is placed into a Lewis negative recipient, the kidney itself may continue to produce the Lewis antigen and initiate immune-mediated rejection, even though the donor and recipient were considered excellent tissue antigen matches.

CONCLUSION

The Lewis^a antigen can be purified easily by a combination of extraction and immunoaffinity chromatography. This novel combination of techniques gave reliable yields of the Lewis^a glycospinoglipid with minimal expenditure of reagents and time. Lewis^a antigen, purified by this method, can readily be used in an enzyme-linked immunosorbent assay to detect low levels of anti-Lewis antibodies in transplant patients experiencing recurrent rejections. At present, we are developing such a test for routine screening of transplant patients at our institution.

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