Isolation and Identification of Trophoblast Lymphocyte Cross-reactive (TLX) Antigens from Human Lymphocytes*

(Received for publication, November 8, 1988)

In Cheol Kim

From the Division of Biomedical Research, Lovelace Medical Foundation, Albuquerque, New Mexico 87108

It has been proposed that allotypic trophoblast lymphocyte cross-reactive (TLX) antigens are involved in the maintenance of normal human reproduction. Despite such a potentially important role for TLX antigens, isolation of human TLX proteins has not yet been reported. As an initial step toward elucidation of the structure and function of TLX antigens, we have isolated TLX proteins from Lubrol-solubilized lymphocytes (termed "wTLX") by anti-trophoblast membrane-Sepharose immunoaffinity chromatography. Using Ouchterlony immunodiffusion and immunoelectrophoresis, we identified an immunoprecipitate wTLX antigen which forms a single immunoprecipitation line against absorbed anti-trophoblast membrane. From 17.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analyses, a 35-kDa band was determined to be a major protein band in the immunoaffinity-isolated wTLX fraction, along with multiple minor wTLX bands. These results suggest the possible existence of antigenic polymorphism of TLX, with predominant expression of the 35-kDa wTLX antigen in lymphocytes. The strong staining of the TLX antigens with Coomassie Brilliant Blue and Amido Black indicates they are largely proteins. Co-isolation of \( \beta \)-microglobulin in the immunoaffinity-isolated wTLX pool could imply that the wTLX antigens may be unique class I HLA-like antigens. This possibility has yet to be resolved.

The placenta is a complex endocrine organ which develops only during pregnancy and is composed of cells of both fetal and maternal origin. An important property of the placenta is that it, along with the fetus, is not rejected by the mother's immune system, although they are genetically different from the maternal host. Because of its complexity, the exact nature of the immunological and physiological interactions between mother and fetus during normal and pathological pregnancies remains an unsolved question (1).

One of the plausible mechanisms proposed to explain the maintenance of normal human pregnancies is the critical involvement of trophoblast-lymphocyte cross-reactive (TLX) alloantigens, which has been put forth by McIntyre and Faulk (2-4). The TLX antigens, which are closely associated with the HLA system (2), are membrane proteins which are expressed commonly on trophoblast and lymphocyte surface membranes. It is not known whether rTLX (TLX in trophoblast) and wTLX (TLX in white blood cell) proteins are structurally identical or not. However, the TLX hypothesis is based on the assumption that unless the structure of TLX antigens of the male is significantly different from that of the mating female, the mother cannot produce anti-TLX antibodies which recognize and protect the blastocystic trophoblast during pregnancy (2-4). Therefore, the TLX antigens must be allotypic to provoke a maternal immune response. Accordingly, the resultant maternal anti-TLX antibodies would serve as protective blocking antibodies both by promoting allogeneic recognition and by blocking anti-paternal immune responses. Failure to produce these blocking antibodies due to TLX sharing between mating couples would result in a spontaneous abortion (2-4). Operation of a similar mechanism involving blocking antibodies has been proposed to explain protection and growth of tumor cells (5).

Therefore, in order to study the biochemical and immunological roles of the TLX antigens during normal human pregnancy, elucidation of the nature and structure of TLX antigens is essential. Although identification of three TLX antigens on human lymphocytes has been reported by Stern et al. (5) and also by Kim and Sabourin (6), none of the TLX antigens has been isolated or characterized. In this communication, we report for the first time the isolation of highly purified wTLX antigens from Lubrol-solubilized human lymphocytes. The potential significance of the TLX antigens in human pregnancy is discussed.

EXPERIMENTAL PROCEDURES

Materials—Rabbit anti-normal human serum (NHS) and anti-\( \beta \)-microglobulin were purchased from ICN Biomedicals. Rabbit anti-NHS IgG fraction was purchased from United States Biochemical Corp. Goat anti-rabbit IgG horseradish peroxidase conjugate, protein molecular weight markers, and nitrocellulose paper were obtained from Bio-Rad. Lubrol PX, NHS-agarose affinity gels, phenylmethanesulfonyl fluoride (PMSF), and neuraminidase were from Sigma.

Preparation of Trophoblast Membrane (TM) from Human Term Placenta—Microvillous TM was prepared from term placenta according to a slightly modified procedure of Smith et al. (7), and the details were described previously (6). The purified TM preparation was used as an immunogen to produce anti-TM serum and also for solubilization in 0.5% Lubrol.

Preparation of Anti-TM Serum and Subsequent Preparation of Anti-TM from Anti-TM Using Solid Phase Affinity Chromatography—Preparation and production of rabbit antisera to TM (anti-TM) was described previously (6). The TM preparations used for immunization in this study were from several pregnant women. Portions of the unsolubilized anti-TM IgG fractions (396 mg) were conjugated to CNBr-Sepharose (8 g) to prepare anti-TM immunoadfinity gels.

Since the anti-TM serum contains minor antibody populations which cross-react with NHS proteins (e.g., anti-albumin), these cross-
reactive antibodies were removed by using albumin- and α₂-macroglobulin-Sepharose affinity columns (6). In addition, NHS-Sepharose affinity gel column chromatography was also introduced to further eliminate any anti-NHS populations in the anti-TM serum. Affinity column chromatography was repeated three times for each gel column. The flow-through volumes from these affinity columns were pooled and precipitated at 50% ammonium sulfate saturation. After centrifugation, the pellet was suspended in 0.05 M Tris buffer, pH 7.4, and dialyzed against the same buffer. The absorbed antisem was designated “anti-TMα.” The anti-TMα was highly specific for TM-bound proteins (6) and was used as an immunological reagent for the detection and identification of the TLX antigens in lymphocytes. The protein concentration of anti-TMα was 45.5 mg/ml.

Preparation of Human Lymphocytes—Buffy coats (about 30-35 ml) were obtained from the local Blood Services United. A buffy coat was diluted with phosphate-buffered saline (PBS) to 75 ml. Each 15 ml of the diluted buffy coat was layered on Ficoll-Paque (15 ml). The tubes were centrifuged at 400 × g for 30 min at 25 °C, and a yellowish, thick interface (buffy layer) was collected, pooled, and diluted with an equal volume of PBS. The cell suspension was centrifuged at 400 × g for 10 min, followed by resuspension in PBS. The Ficoll-Paque centrifugation was repeated. The interface was again collected and brought to 35 ml with PBS. The suspension was centrifuged at 400 × g for 10 min. The yellowish pellet was resuspended in PBS and centrifuged at 200 × g for 10 min. Washing of the cell pellets was repeated once more. Finally, lymphocyte pellets were resuspended in 1 ml of PBS, counted with a hemocytometer, and kept frozen at −20 °C for stock.

Isolation of the wTLX Antigens from Solubilized Lymphocytes by Immunofinity Chromatography—Thawed lymphocytes were suspended in 15 ml of 0.5% Lubrol buffer (0.5% Lubrol, 1 mM EDTA, 50 mM Tris buffer, pH 8.0) containing 0.1 mM PMSF and solubilized by stirring overnight at 4 °C. After centrifugation at 20,000 × g for 10 min, the supernatant (32 ml containing a total of 32 mg of protein) was mixed with 10 ml of anti-TM IgG-Sepharose gel and left at 4 °C overnight. The gel was poured onto a column and thoroughly washed with 0.1% Lubrol buffer. Bound antigens were eluted with 1 N acetic acid containing 0.5 M NaCl and 0.1% Lubrol buffer. Eluates were immediately neutralized with 2 M Tris. The immunofinity chromatography was repeated twice more with the flow-through fractions. Eluates were then pooled and applied to an anti-NHS IgG-Sepharose immunofinity column (bed volume: 10 ml) to remove any contaminating NHS components. The immunofinity chromatography was repeated three times with the flow-through fractions. Finally, the flow-through fractions were pooled and concentrated in an Amicon membrane concentrator (YM 5 membrane) and termed the “immunoaffinity-isolated wTLX antigens.”

Immunological Analysis—Double immunodiffusion and immunoelectrophoresis (IEP) were performed as described previously (6). Throughout, 0.5% Lubrol was included in 1% agarose solution. The immunoelectrophoresis were washed routinely with 0.9% NaCl and water, dried, and stained with Coomassie Brilliant Blue.

Immunoblot Analysis (Western Blot)—Immunoblot analyses were performed as described previously (6, 8). Briefly, 17.5% SDS-PAGE was performed under reducing and nonreducing conditions, and proteins were electrostrically transferred to nitrocellulose papers. Blotted nitrocellulose paper was blocked with 3% gelatin overnight and then treated with appropriately diluted rabbit antiserum for 1 h at room temperature. The antibody-treated nitrocellulose paper was incubated with 1000-fold diluted goat anti-rabbit IgG horseradish peroxidase conjugate for 1 h at room temperature. The color reaction was performed with 4-chloro-1-naphthol and hydrogen peroxide.

Neuraminidase Treatment—The immunofinity-isolated wTLX pool (0.67 mg/ml) was treated at 37 °C overnight with 1 mg of neuraminidase (1.8 units) in 0.05 M acetate buffer, pH 5.5, containing 4 mM CaCl₂. Human placental alkaline phosphatase was used as a sialic acid-containing marker protein to monitor the occurrence of desialylation during the enzyme treatment.

Protein Determination—Proteins were determined with the BCA protein assay procedure (9) in the presence of 0.1% Lubrol, with bovine serum albumin as the standard. The concentration of albumin was determined with a 1% extinction coefficient of 0.6 to 280 nm.

RESULTS

Immunofinity Isolation of wTLX Antigens from Solubilized Human Lymphocytes—Since the nature of TLX is not well defined and monospecific anti-TLX is not available, a practical approach for the isolation of wTLX antigens from lymphocytes is to utilize an anti-TM immunoaffinity technique. Therefore, a mixture of the lymphocytes of 16 donors was initially solubilized in Tris buffer containing 0.5% Lubrol. During the solubilization process, 0.1 mM PMSF and 1 mM EDTA were included to minimize proteolysis. TLX antigens were isolated using an anti-TM Sepharose immunoaffinity column. Eluates were then applied to an anti-NHS IgG Sepharose immunoaffinity column to reduce or remove any contamination by NHS proteins. For immunofinity chromatography procedures, the Lubrol concentration in the buffer was lowered to 0.1% to minimize interference with SDS-PAGE. The yields of the immunoaffinity-isolated wTLX pools were 675 μg and 530 μg from 2.89 × 10⁵ and 3.89 × 10⁷ lymphocytes, respectively.

Ouchterlony Double Immunodiffusion—In Ouchterlony immunodiffusion of the immunoaffinity-isolated wTLX against anti-TMα, only a single immunoprecipitation line was detected due to the presence of a major wTLX antigen in the immunoaffinity-isolated fractions (wells 1, Fig. 1A). The anti-TMα did not react with NHS (wells 2, Fig. 1A), indicating the high specificity of the anti-TMα for the wTLX antigen. In further experiments against a panel of antisera to various human serum proteins, no immunoprecipitation lines were detected (results not shown). This suggests that the wTLX antigen is a lymphocyte-specific protein.

To determine the purity of the wTLX preparations, Ouchterlony immunodiffusion was performed against anti-NHS. As shown in Fig. 1B (wells 1), a blured, weak immunoprecipitation line was detected between the wTLX pool and anti-NHS, indicating that the wTLX pool is contaminated with a

![Fig. 1](https://example.com/fig1.png)
trace of NHS protein. The nature of the cross-reacting NHS protein has not yet been identified.

Formation of an immunoprecipitation line between anti-β′m and the wTLX pool indicates that the immunoaffinity-isolated wTLX pool contains β′m (wells 3 in Fig. 1B). However, the intersection of two immunoprecipitation lines between wells 2 (anti-TMa) and wells 3 (anti-β′m) indicates that the wTLX recognized by anti-TMa is not β′m, and also that anti-TMa does not contain anti-β′m antibody.

In Ouchterlony immunodiffusion of the immunoaffinity-isolated wTLX pool (wells 2 in Fig. 1C) and the immunoaffinity-isolated TM pool against anti-TMa (see wells 3 in Fig. 2C), complete fusion was observed between the wTLX immunoprecipitation line of wells 2 (containing the immunoaffinity-isolated wTLX) and one of the three immunoprecipitation lines of wells 3 (containing the immunoaffinity-isolated TM). The tTLX line (present in TM) belongs to one of the three immunoprecipitation lines formed between anti-TMa and immunoaffinity-isolated TM (see wells 3 in Fig. 1C). This suggests that the lymphocyte membrane wTLX is antigenically identical with a tTLX antigen present in the immunoaffinity-isolated TM.

**Immunoelectrophoresis (IEP)—**An immunoprecipitation arc was detected from IEP of the immunoaffinity-isolated wTLX pool against anti-TMa, anti-β′m, and anti-NHS (see Fig. 1D). A weak reaction line between the wTLX and anti-NHS indicates that the immunoaffinity-isolated wTLX pool is contaminated with a trace amount of an NHS protein (see trough c). The IEP experiments further confirm the presence of β′m in the immunoaffinity-isolated wTLX antigen pool. The immunoelectrophoretic mobility of the wTLX antigen was slightly faster than that of β′m.

**SDS-PAGE Profiles—**For identification of the numbers and subunit molecular sizes of proteins found in the immunoaffinity-isolated wTLX pool, 17.5% SDS-PAGE was performed under both reducing and nonreducing conditions. Under reducing conditions, a band with a molecular mass of 35 kDa was the major and predominant protein band in the wTLX pool (see an arrow marked in lane 2, Fig. 2). Several additional protein bands were also visible in the wTLX pool, which indicates that additional purification steps are necessary to obtain a pure form of the wTLX antigen.

It appears that the 35-kDa band is also a major protein band in Lubrol-solubilized TM (lane 3, Fig. 2). But it is not known whether the major 35-kDa band in TM is a trophoblast membrane TLX. The observation that a 35-kDa band was not a major protein band in solubilized lymphocytes (lane 4, Fig. 2) suggests that the density of the 35-kDa wTLX band is probably very low on lymphocyte membranes. The discrepancy in the size of the wTLX antigen between 35 kDa (this study) and 40 kDa (the previous study, Ref. 6) is probably due to the use of 17.5% and 15% polyacrylamide gel concentrations, respectively.

Judged by 17.5% SDS-PAGE of the wTLX pool under nonreducing conditions, the wTLX antigens underwent aggregation by forming multiple, diffused, high molecular mass bands (see arrows in lane 2 in Fig. 3, A and B). This suggests that the wTLX antigens are probably hydrophobic proteins which undergo polymerization by disulfide cross-linking. In addition, a 31-kDa band consistently appeared under nonreducing conditions (see lane 2 in Fig. 3A). The occurrence of the 31-kDa band is probably due to the formation of an aggregate of contaminant, low molecular mass membrane proteins. Since the 31-kDa band did not react with anti-TMa (lane 2, Fig. 3B), it was not related to TLX antigens.

**Western Blots—**From Western blots of wTLX with anti-TMa, we observed that a major anti-TMa-reactive band is located at the 35-kDa region (see arrows in lanes 2 and 4 in Fig. 4) and also that two additional, minor bands were present just above and below the 35-kDa band (lane 3 in Fig. 3, A and B, and lanes 2 and 4 in Fig. 4; minor bands are not well reproduced in the photographs). The multiple, minor TLX bands were not recognized by anti-NHS (lane 6 in Fig. 4). This suggests a possible polymorphic or allotypic nature of the wTLX antigens in lymphocytes. In addition, absence of binding by anti-TMa to NHS proteins (lane 5 in Fig. 4) indicates that anti-TMa is indeed highly specific for TLX antigens.

Immunoblot assays of Lubrol-solubilized, crude lymphocyte extracts (a mixture of lymphocytes obtained from 16 donors) with anti-TMa revealed the 35-kDa band to be a major and predominant TLX band and also revealed five additional minor TLX bands (see arrows in lane 4 in Fig. 3B). Therefore, it appears that the 35-kDa band is the major TLX antigen, and also that there are multiple, minor TLX antigens on lymphocyte membranes. Further detailed studies are necessary with allospecific antibodies and a large variety of healthy donors’ lymphocytes to prove whether or not these multiple,
minor bands reflect TLX allelic variants or TLX subsets.

Immunoblot assay of wTLX with 250-fold diluted anti-NHS was negative except for the 55-kDa rabbit IgG γ-chain band (lane 6, Fig. 4; see below). Although contamination by an NHS component in the wTLX pool was detected from immunodiffusion (wells 1, Fig. 1B) and IEP (trough c, Fig. 1D), we were unable to detect and identify the anti-NHS-reactive antigen from Western blots. The failure to detect this NHS protein is possibly due to its low concentration in the wTLX pool, or to considerable destruction of its antigenicity during Western blotting (e.g. by SDS).

Immunoblot analysis of wTLX with anti-βm solution detected a weak band at 11.7 kDa, further confirming the presence of βm in the wTLX pool (see arrow in lane 7, Fig. 4). Despite strong immunoreactivity of anti-βm with wTLX in immunodiffusion (wells 3, Fig. 1B) and IEP (trough b, Fig. 1D), the immunoreactivity of βm with 100-fold diluted anti-βm was very weak in Western blots. This suggests that the antigenicity of βm was to a great extent destroyed during Western blotting.

A direct immunoblot assay of wTLX-blotted nitrocellulose with goat anti-rabbit IgG peroxidase conjugate antibody (without prior treatment with first antibody) revealed a binding activity in the 55-kDa region (see lane 8, Fig. 4), indicating the presence of rabbit IgG in the wTLX pool. Therefore, the presence of the 55-kDa band was due to the heavy (γ) chain of rabbit IgG, which leaked during rabbit anti-TM IgG-Sepharose immunoadfinity chromatography. By Ouchterlony immunodiffusion against goat anti-rabbit IgG, the presence of rabbit IgG in wTLX was also confirmed (results not shown). Thus, the 55-kDa band detected in wTLX by anti-TM (lane 4, Fig. 4), anti-NHS (lane 6, Fig. 4), and anti-βm (lane 7, Fig. 4) is due to the presence of contaminating rabbit IgG γ-chain, not due to another form of wTLX antigen.

Analysis of Neuraminidase-treated wTLX—17.5% SDS-PAGE indicated no difference in the electrophoretic mobility of neuraminidase-treated wTLX. From Ouchterlony immunodiffusion and Western blot analysis of neuraminidase-treated wTLX antigen, there was no alteration in the antigenicity of the 35-kDa band (results not shown). This suggests that the wTLX does not contain sialic acid, or the content is very low.

**DISCUSSION**

During human pregnancy, a complex chain of physiological and immunological processes must occur to protect the fetus which expresses paternal gene products. Any perturbation in these processes during pregnancy would result in pathological pregnancies, e.g. spontaneous abortions. To prevent such pregnancies, it is necessary to understand the biochemical and immunological mechanisms of pregnancy, particularly at the trophoblast level where direct fetomaternial interaction occurs. Recently, it has been proposed that allotypic TLX antigens are involved in the maintenance of normal pregnancy by modulating maternal rejection of the fetus (2-4). Since TLX antigens have yet to be isolated, investigation of their biological roles in human pregnancy has not been feasible. However, clinical studies have supported a potential role for the TLX antigens in achieving successful pregnancies, particularly in recurrent aborters. Multiple transfusions of husband's (10-12) or third party donors' pooled lymphocytes (13, 14) to spontaneously recurrent aborters increased the chances of pregnancy and delivery of normal babies. These clinical observations suggest that the maternal immune response was probably stimulated by TLX alloantigens in the husband's or third party's lymphocytes, to produce anti-TLX blocking antibodies. Therefore, it appears that TLX antigens play an important regulatory role in influencing genetic, immunolog-
Trophoblast Lymphocyte Cross-reactive (wTLX) Antigens

For reasons described above, understanding the nature and structure of TLX antigens becomes an essential step to initiate a thorough investigation of fetomaternal interaction during normal and pathological pregnancies. Subsequently, by utilizing TLX antigens or anti-TLX antibodies as pregnancy marker proteins for normal fetal development, possible causes of spontaneously recurrent abortions or other pathological pregnancies can be studied at the molecular level. Although the identification of lymphocyte wTLX antigens as proteins of 65 kDa and 55 kDa has been reported by Stern et al. (5), these TLX antigens have not yet been isolated. Therefore, in this study, a mixture of 16 donors' lymphocytes was used for the isolation of wTLX antigens, and antiserum to TM used in this study was produced against several TM preparations. Thus, the antibodies bind and recognize most of the allelic variants of TLX antigens. Therefore, the detection of several minor TLX bands in lymphocytes suggests a polymorphism in wTLX antigens similar to that of the major histocompatibility complex antigens (15).

One of the unanswered questions in this study is the exact status of β4-microglobulin, which normally associates with class I HLA antigens (15). Despite the absence of anti-β4m antibody in our anti-TM serum (Ref. 6, and also see Fig. 1B), β4m was consistently co-isolated in the immunoaffinity-isolated wTLX fractions. The reason could be either due to nonspecific contamination from solubilized lymphocytes during immunoaffinity chromatography or due to a strong structural interaction between the TLX antigens with β4m on lymphocyte membranes. The smaller size (35 kDa) of the major wTLX antigen indicates that it is not a common class I HLA alloantigen of 45 kDa (15). Association of the 35-kDa wTLX antigen with β4m could qualify it as a novel class I HLA antigen. Therefore, it remains to be confirmed whether the 35-kDa wTLX antigen indeed forms a protein complex with β4m in situ on lymphocyte membranes.

Finally, our success in the isolation of the 35-kDa TLX antigen will provide a basis for future detailed structural studies and the mechanism of its role in human reproduction. Proof of its biochemical and immunological importance in the maintenance of normal human pregnancy would facilitate the development of TLX-based synthetic peptides, instead of lymphocytes, as therapeutic immunogens for spontaneously recurrent aborters.

REFERENCES