Purification and Characterization of Papain-Solubilized HLA Antigens From Human Platelets

By Jon P. Gockerman and William Jacob

Human platelet membranes were isolated by the hypotonic glycerol lysis technique from donors who had their HLA antigens identified on lymphocytes by lymphocytotoxicity. The HLA antigens of the platelets were solubilized by papain treatment of the isolated membranes. The solubilized material retained antigenic activity and specificity. This crude antigenic material was further purified by column chromatographic techniques. On Sephadex chromatography the antigenic material had an estimated molecular weight of 40,000 ± 3500 d (1 SD). It contained material that reacted immunologically with an antibody specific for β2-microglobulin. Further purification of this material by DEAE-cellulose chromatography followed by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) showed it to be composed of two pieces. One piece had a molecular weight of 12,000 d, and the larger piece had a molecular weight of 26,000 d. The purification procedure resulted in a 540-fold increase in HLA-A2 specific activity, with a yield of 0.26 mg per 6 X 10¹¹ platelets. β2-microglobulin co-chromatographed with the HLA antigen activity on exclusion and ion-exchange chromatography and migrated with HLA antigen activity on non-SDS-PAGE gels. The 12,000-d band on SDS-PAGE gels was believed to represent β2-microglobulin. These results suggest that HLA antigens of platelets and lymphocytes are similar in structure when solubilized by papain and that platelets can be used as a source of HLA antigens for further structural analysis.

The major histocompatibility complex is a term used to describe a region of chromosome number 6 in man that influences an array of immune processes including allograft survival. The HLA complex is a part of this major histocompatibility complex, and it determines the serologically defined and lymphocyte-defined cell surface antigens. The serologically defined HLA antigens are present on most human cells, including the platelet, and are determined by three separate genes (HLA-A, -B, -C) that code for approximately 50 different alleles. The product of each allele (i.e., HLA antigen) can be serologically detected on the lymphocyte surface. The HLA antigens from loci A and B are best characterized by serological techniques. Considerable progress has been made in the isolation, purification, and structural analysis of the HLA antigens obtained from human lymphocytes grown in tissue culture and from a similar set of antigens (H-2) on mouse lymphocytes. These studies have shown that when HLA antigens from cultured human lymphocytes are solubilized by papain, a 48,000-d piece is obtained that is composed of a 12,000-d polypeptide identical to β2-microglobulin.
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and linked noncovalently with a 34,000-d glycopeptide. The 34,000-d glycopeptide carries the serological specificity of the HLA antigen that is independent of its carbohydrate side chain and β2-microglobulin but is dependent on the amino acid sequence and the tertiary structure of the glycopeptide chain. Each HLA gene codes for an individual glycopeptide that can be isolated from papain-purified lymphocyte antigens by ion-exchange chromatography or isoelectric focusing. Solubilization of the HLA antigens by a nonionic detergent results in a piece with larger molecular weight (44,000 d), along with the 12,000-d β2-microglobulin. When the 44,000-d piece is subjected to papain digestion, a 34,000-d piece is produced that does not bind detergent. In the process of papain digestion two peptides are released, the first being hydrophilic and the second hydrophobic. Further, amino acid sequencing studies show that the 44,000-d HLA antigen is composed of a C-terminal hydrophilic group of amino acids inside the lymphocyte, followed by a group of hydrophobic amino acids within the cell membrane, and this is followed by the hydrophilic 34,000-d papain-soluble piece extending out of the cell membrane. This view appears to be confirmed by the ability of lactoperoxidase-catalyzed iodination to label the HLA antigens of the everted lymphocyte plasma membranes, showing that the HLA molecule extends through the membrane. The general structural outline is followed when detergent-soluble HLA antigen is incorporated into phospholipid vesicles (liposomes). These studies plus recent reports showing the importance of the HLA antigens in cooperative immunologic cell interactions suggest that a cell may be able to receive an internal signal when binding occurs to the outer HLA antigens.

The platelet appears to possess the same serologically determined HLA antigens as the lymphocyte, but in lesser quantities. There have been some attempts to solubilize and isolate the human platelet HLA antigen using nonionic detergent NP-40, 3-M KCl, and a preliminary study has used papain. Antigenically active HLA antigen has been solubilized by 3-M KCl, NP-40, and papain from human platelets. Individual HLA antigens can be separated by preparative polyacrylamide gel electrophoresis. NP-40 solubilization results in material with an estimated molecular weight greater than 200,000 d that dissociates on polyacrylamide gel electrophoresis (PAGE) in sodium dodecyl sulfate (SDS) into two major components of 45,000 and 90,000 d. These initial attempts at purification suggest some molecular weight differences between platelet and lymphocyte HLA antigens. Further, these studies revealed an extremely poor yield of HLA antigen from platelets. There has been no confirmation that the structure of the HLA antigen on the platelet is similar to that on the lymphocyte.

The cell surface proteins of the platelet appear to be important to normal platelet function. The exact role the HLA antigen plays in platelet function is unclear, although antibodies directed to HLA antigens can trigger platelet aggregation. A recent study has shown that increased levels of platelet cyclic AMP allowed more HLA antigens to be solubilized by 3-M KCl extraction. Further, although β2-microglobulin has been measured in the platelet, its surface distribution and relation to the HLA antigens are unknown. In an attempt to understand the structural relationships between HLA antigens on different cells and to explore the structural-functional roles of the platelet HLA antigens, human platelet HLA antigens were solubilized, purified, and characterized.
MATERIALS AND METHODS

Chemicals

Papain was obtained from Sigma Chemical, St. Louis, Mo., Sephadex G-100 from Pharmacia, Piscataway, N.J., DEAE-52 and CM-52 from H. Reeve Angel, Clifton, N.J., molecular weight standards from Worthington, Freehold, N.J., Pharmacia, and Sigma. The polyacrylamide gel electrophoresis reagents were obtained from Bio-Rad, Richmond, Calif. All other chemicals were obtained from Fisher Scientific, Fair Lawn, N.J., and were of the highest purity available. All glassware was siliconized with Siliclad, Clay Adams, Parsippany, N.J.

Cells and Preparation of the Membranes

Platelets were obtained fresh from normal male donors by standard blood bank techniques allowing four units of platelets to be harvested per one bleeding.25 The platelets were allowed to disaggregate in 30 ml of donor plasma, then to sit 12 hr at room temperature in 45-ml polycarbonate test tubes. Platelet-rich plasma was then decanted from any red cells that had settled to the bottom of the tube. The platelets were pelleted by centrifugation, washed twice with a solution of 0.15-M NaCl, 0.001-M Na2EDTA, and 0.01-M Tris-HCl (pH 7.5), and subjected to hypotonic lysis after glycerol loading with some modifications of a previously described technique.29 The platelets, after washing, were layered on 35 ml of a 0%-40% linear glycerol gradient and centrifuged at 1500 g for 45 min and then at 5000 g for all at 4°C. The pellet formed was collected and subjected to hypotonic lysis by mixing with 0.01-M Tris-0.25-M sucrose (pH 7.5) on a vortexer for 3 min. This material was then sonicated (setting 6, microtip, Sonifer Cell Disruptor, model W 185, Heat Systems-Ultrasonics, Plainview, N.Y.) for 2 min, being kept in an ice bath during the procedure. The material was layered on 20 ml of a 27% (w/v) sucrose cushion (density 1.106) and centrifuged at 63,500 g for 4 hr at 4°C. A faint band appeared in the middle of the tube and was harvested with a siliconized Pasteur pipette. This membrane suspension was then pelleted by centrifugation at 105,000 g for 1 hr. A check of the membrane pellet by electron microscopy showed primarily platelet membranes, as with the original procedure, but the addition of sonification resulted in an improved membrane yield. The membranes were resuspended in 0.01-M Tris (pH 8.0) and used immediately or stored for no longer than 7 days in a liquid nitrogen vapor.

Solubilization of HLA Antigens by Papain

The purified membranes suspended in 0.01-M Tris (pH 8.0) were made to 5-mM cysteine with 100-mM cysteine. After warming in a water bath for 10 min at 37°C, four units of papain per milligram of protein as determined by the Lowry technique using bovine serum albumin as a standard were added.30 The suspension was placed in a shaking water bath at 37°C for 1 hr. The reaction was stopped by placing it in an ice bath and immediately adding an appropriate volume of 130-mM sodium iodoacetate to give a final concentration of 6.5 mM. An alternative method to stop the papain activity was to pass the reaction mixture over a CM-52 column previously equilibrated with 4-mM sodium phosphate (pH 6.0) and then eluted with this buffer. Two times the void volume was collected that contained all the HLA activity.

Purification of Papain-Solubilized HLA Antigen

All purification procedures were carried out at 4°C in a cold room. The solubilized material was dialyzed for 12 hr against 0.15-M NaCl-0.01-M Tris (pH 8.0) and then applied to a G-100 Sephadex column (3 ml on a column 1.6 × 26 cm). The column was eluted with 0.15-M NaCl-0.01-M Tris (pH 8.0) buffer, and 1-ml fractions were collected. Each fraction was analyzed for protein by its absorbency at 280 nm and tested for its inhibitory activity as described below. The tubes showing inhibitory activity were pooled and dialyzed for 18 hr against 0.005-M Tris-0.005-M sodium phosphate (pH 8.0) and then applied to a DEAE column (0.9 × 10 cm) previously equilibrated with the same buffer. The column was eluted with 250 ml of linear gradient from 0.005-M Tris–sodium phosphate (pH 8.0) to 0.1-M Tris–sodium phosphate (pH 5.6). Each fraction was analyzed for protein by its absorbency at 280 nm and its inhibitory activity. Aliquots were saved through the purification for titration of inhibitory activity and for gel electrophoresis.
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HLA Activity Assay

HLA specificity for platelet donors was determined by a standard microlymphocytotoxicity assay using HLA antisera obtained from the Transplantation Immunology Branch, NIAID, NIH. HLA antigenic activity solubilized from platelets was detected by inhibition of anti-HLA antibody on the lymphocytes of the platelet donor by the solubilized-platelet-HLA-containing material. This was carried out using standard procedures, except that the lymphocytes were purified from defibrinated blood so as to decrease platelet contamination of the lymphocyte preparation. The amount of soluble antigen that would inhibit killing of 50% of the lymphocytes in the presence of an antibody previously titrated to give 100% kill was called one ID unit.

The inhibitory assay was performed by mixing in a Microtiter plate under oil 1 μl of anti-HLA antibody causing 100% cell kill with 1 μl of antigenic material (or buffer as a control) and incubating at 37°C for 45 min. One microliter of lymphocytes (2500/cu mm) was added and incubated 30 min at 20°C. The lymphocytes were then washed with 10 μl of veronal-buffered saline; 5 μl of rabbit complement were then added, and the mixture was incubated 60 min at 20°C. Trypan blue dye was added, and cell viability was determined by dye exclusion using an inverted phase microscope.

There was variation in ID₉₀ titers depending on the antibody and lymphocytes used. But using the same antibody and lymphocytes the ID₉₀ titers were repeatable, and this is the procedure used in reporting titers in this article. A similar titration procedure was used for β₂-microglobulin using a rabbit antihuman β₂-microglobulin obtained from DAKO, Accurate Chemical and Scientific, Hicksville, N.Y. A broad-spectrum equine antilymphoblastic globulin was used as a positive control (gift of Dr. D. Kimball, Walter Reed Army Medical Center).

PAGE

PAGE was performed in 7- × 75-mm glass tubes with and without SDS using a previously described procedure. Gels were prepared from a stock solution of 30% (by weight) acrylamide and 0.8% N,N'-bis-methylene-acrylamide. The separating gel was 0.375-M Tris-HCl (pH 8.9) and 0.1% SDS, and the stacking gel was 0.0619-M Tris-HCl (pH 6.8) and 0.1% SDS. Gels were polymerized by the addition of 5 μl of tetramethylenehexahylenediamine (TEMED) and 0.1 ml of a 10% solution of ammonium persulfate to 10 ml of solution. The electrophoresis buffer was 0.025-M Tris-HCl, 0.192-M glycine (pH 8.3), and 1% SDS. The samples of platelet antigenic material were dialyzed against water and concentrated by lyophilization. The material was resuspended in a sample buffer containing 10% glycerol, 5% mercaptoethanol, 2% SDS, and 0.0619-M Tris-HCl (pH 6.8). This mixture was allowed to stand at 20°C for 12 hr or at 37°C for 2 hr and gave reproducible gel patterns. The sample contained 10–60 μg of protein in a volume not exceeding 50 μl. It was layered on 60-mm 12% acrylamide separating gel with a 5-mm 3% acrylamide stacking gel. The current was adjusted to 1 mamp per gel until the tracking dye entered the separating gel, and then the current was readjusted to 2.5 mamp per gel until the tracking dye was several millimeters from the bottom of the tube. The buffer chamber was maintained at 4°C by a cooling jacket. The position of the tracking dye was marked with a thin piece of wire. The gels were fixed for 24 hr in 12.5% trichloroacetic acid at 20°C, then stained for 2 hr with Coomassie blue at 20°C, and then destained using a Bio-Rad diffusion destainer in 7% acetic acid. In gels using a non-SDS-PAGE system, the buffers were the same as described, except that SDS was omitted; the sample preparation was the same, except that SDS, mercaptoethanol, and the incubation period were omitted; and a 3% acrylamide stacking gel was used with a 7% acrylamide separating gel.

Ouchterlony Gel Diffusion

Ouchterlony gel diffusion was performed by standard techniques using 2-mm slices of PAGE gels overlaid with agarose gel.

Platelet Immunofluorescence

Platelet immunofluorescence was performed using a double-antibody technique: rabbit anti-β₂-microglobulin and swine antirabbit IgG conjugated with fluorescein-isothiocyanate isomer I (DAKO). The procedure used was a tube technique previously described for detection of platelet isoantibodies. The immunofluorescence was observed and photographed on a Zeiss Photomicroscope III (Carl Zeiss, New York, N.Y.).
RESULTS

Solubilization of HLA Antigens From Platelet Membranes

Papain treatment of platelet membranes resulted in the solubilization of HLA antigenically active material. By using different donors of platelets, papain treatment resulted in solubilization of HLA inhibitor activity for HLA-A1, 2, 11, W32, and HLA-B7, 8, 12, 18, W17, 27, W35. The material solubilized from one platelet source would not inhibit antibody-mediated lymphocytotoxicity against HLA antigens not represented on the surfaces of the lymphocytes of the platelet donor. But the solubilized material did inhibit activity on lymphocytes having the same HLA-A and HLA-B antigens as were present on lymphocytes of the platelet donor. The papain-solubilized HLA antigen did not inhibit a broad-spectrum antilymphoblastic globulin.

Our initial studies compared the amount of HLA antigenic material solubilized from intact platelet membranes and that obtained from isolated membranes. When isolated platelet membranes were treated with papain there was a twofold increase in total HLA inhibitory activity and an 11-fold increase in specific activity, as compared with using whole platelets. There was good reproducibility in isolation of the platelet membranes, resulting in 2.7 ± 0.9% (1 SD) of the total platelet protein being present in the isolated membranes.

Figure 1 shows the results of inhibitory activity when different doses of papain were used. There was an optimal range between 1 and 4 units of papain per milligram of protein where the maximum soluble HLA inhibitory activity was obtained. Only slight variability was noted in the amount of protein solubilized by papain treatment of the isolated membranes, i.e., 18 ± 0.8% (1 SD) of the protein of the isolated platelet membrane was solubilized.

In initial attempts to purify the HLA antigens the papain-solubilized material was concentrated by ultrafiltration, resulting in significant loss of inhibitory activity. Therefore, it was desirable to keep the initial solubilized antigen in a small volume, and this was achieved by inhibition of papain activity by the addition of sodium iodoacetate rather than passage of the material over a CM-52 column.

Fig. 1. Relation of papain concentration to the amount of solubilized HLA-A2 antigen. Platelet membranes were incubated at 37°C for 1 hr with various concentrations of papain added per milligram of platelet membrane protein. The papain was inhibited by sodium iodoacetate and then centrifuged at 105,000 g for 1 hr. The supernatant was titrated for HLA-A2 inhibitory activity. This graph is the mean of three separate studies.
There was no difference in the yield of inhibitor activity obtained using either method of eliminating papain activity.

In the standard procedure the platelet membranes isolated by glycerol loading and hypotonic lysis and then treated with papain resulted in 1% of the total platelet protein being solubilized. In general, 8 units of platelets (6 X 10¹¹ platelets) yielded 10–20 mg of papain-solubilized material containing HLA inhibitory activity.

**Purification of Platelet HLA Antigens**

Figure 2 shows the results of chromatographing the papain-soluble platelet antigen over Sephadex G-100. The material containing the HLA antigen activity comes out in the 40,000 ± 3500-d (1 SD, N = 7) range and co-chromatographs with β₂-microglobulin activity. Although only HLA-A2 and β₂-microglobulin inhibitory activities are depicted on the graph, the other HLA activities are contained in the same elution volume as the β₂-microglobulin activity, although the peak activity differed slightly from that of the HLA-A2.

Figure 3 depicts the chromatograph resulting when the pooled G-100 Sephadex HLA antigen material is eluted from a DEAE column with a linear salt gradient. The HLA-A2 activity comes off at the same time as the β₂-microglobulin activity, but the β₂-microglobulin activity continues for several more tubes. The area with β₂-microglobulin activity beyond the HLA-A2 activity is where the other HLA antigens elute, i.e., there is separation of the different HLA-antigen serological specificities.

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Fig. 2. Elution profile on a column (1.6 X 26 cm) of Sephadex G-100 of the material solubilized from treatment of isolated platelet membranes with papain. The column was eluted with 0.15-M NaCl and 0.01-M Tris (pH 8.0) after a 3.1-ml sample was applied that had been dialyzed against this buffer. An upward flow technique was used, and 1-ml fractions were collected. The procedure was done at 4°C. The inhibition assay was performed immediately on completion of the fractionation.
Fig. 3. Elution profile on a column (0.7 X 10 cm) of DEAE-cellulose at 4°C of material showing inhibition of anti-HLA-A2 antibody from fractionation over G-100 Sephadex column of material solubilized by treatment of isolated platelet membranes with papain. The column was equilibrated initially with 0.005-M Tris-0.005-M phosphate (pH 8.0), and then a linear gradient was established with 0.1-M Tris-0.1-M phosphate (pH 5.8). Fractions of 2 ml were collected, and the inhibition assays were performed immediately on completion of the fractionation.

Figure 4 is a composite picture of the SDS-PAGE gels at different steps in the purification procedure. Gel D is the material containing HLA activity from the DEAE chromatograph. It contains a diffuse low-molecular-weight band, as do all the gels, of about 12,000 d. The other major band is at 26,000 d and can be seen on all the gels. These molecular weight estimates were made using molecular weight standards giving a regression coefficient of 0.982.

Table 1 depicts the degree of enrichment obtained by the isolation and purification procedure outlined. The purification procedure resulted in an overall HLA-A2 enrichment from the isolated membranes of 500-fold, with final yield of 0.26 mg of enriched HLA antigen from 6 X 10^11 platelets. The overall enrichment for β2-microglobulin activity was 137-fold.

β2-Microglobulin Activity

Figures 2 and 3 show that the β2-microglobulin co-chromatographs with the HLA antigen. There was a slight amount of β2-microglobulin inhibitory activity detected when the late protein peak from G-100 Sephadex chromatography (effluent volume 65-95 ml) was concentrated 10-fold (no HLA activity was detected in this material).

Ouchterlony analysis of the G-100 Sephadex HLA pooled antigen yielded a precipitin line with anti-β2-microglobulin. We were unable to detect a precipitin line with a number of different anti-HLA-A2 antibodies at various concentrations. This material was concentrated by vacuum dialysis and then electrophoresed in non-SDS-PAGE gels. The gels were cut into 2-mm slices and either eluted with buffer for 24 hr or overlaid with agarose, and an immunodiffusion assay was run with anti-β2-microglobulin. A precipitin line was formed against two slices, and the
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Fig. 4. SDS-PAGE of each step in the purification of platelet HLA antigens. The procedure was carried out using 3% acrylamide stacking gels and 12% acrylamide separating gels and stained with Coomassie blue as noted in the text. Gel A is from isolated platelet membranes. Gel B is the soluble material obtained after pepsin treatment of isolated platelet membranes. Gel C is the HLA-containing antigenic activity after G-100 chromatography of papain-solubilized platelet membrane material. Gel D is the HLA antigenic material after DEAE-cellulose chromatography of G-100 HLA antigenic material. The molecular weights are based on known standards. The top arrow represents a molecular weight of 26,000 d and the bottom arrow a molecular weight of 12,000 d.

Table 1. Purification of Platelet HLA Antigens

<table>
<thead>
<tr>
<th>Procedure</th>
<th>ID₃₎ Titer (per ml)</th>
<th>Volume (ml)</th>
<th>Total Protein (mg)</th>
<th>Specific Activity (ID₃₅ per mg protein)</th>
<th>Degree of Purification</th>
<th>Percentage Yield</th>
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<tbody>
<tr>
<td>HLA-A2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Platelet membranes prepared by glycerol lysis</td>
<td>31.5 X 10⁷</td>
<td>2.9</td>
<td>54.40</td>
<td>1.68 X 10⁷</td>
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<td>2. (A) Post-papain-solubilized antigen</td>
<td>0.8 X 10⁷</td>
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<td>10.40</td>
<td>0.22 X 10⁷</td>
<td>1</td>
<td>19.1</td>
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<td>(B) Post-papain platelet membrane antigen</td>
<td>0.5 X 10⁷</td>
<td>2.5</td>
<td>24.20</td>
<td>0.05 X 10⁴</td>
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<td>—</td>
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<td>3. Post G-100 Sephadex chromatography</td>
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<td>0.60</td>
<td>35.00 X 10⁸</td>
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<td>5.8</td>
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<td>4. Post DEAE-cellulose chromatography</td>
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<td>0.26</td>
<td>118.46 X 10⁸</td>
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<td>43.3</td>
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<td>b₂-microglobulin</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>1. Platelet membranes prepared by glycerol lysis</td>
<td>21.0 X 10⁷</td>
<td>2.9</td>
<td>54.40</td>
<td>1.12 X 10⁷</td>
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<td>—</td>
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<tr>
<td>2. (A) Post-papain-solubilized antigen</td>
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<td>2.9</td>
<td>10.40</td>
<td>0.17 X 10⁷</td>
<td>1</td>
<td>19.1</td>
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<tr>
<td>(B) Post-papain platelet membrane antigen</td>
<td>5.0 X 10⁷</td>
<td>2.5</td>
<td>24.20</td>
<td>0.52 X 10⁷</td>
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<td>3.58 X 10⁸</td>
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<td>0.37</td>
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Fig. 5. Immunodiffusion pattern of platelet HLA material versus anti-\(\beta_2\)-microglobulin. Platelet HLA antigenically active material from G-100 Sephadex chromatography was electrophoresed on non-SDS polyacrylamide gels. The gels were cut into 3-mm slices and overlaid with agar. Into the central well was placed anti-\(\beta_2\)-microglobulin, and the preparation was incubated for 24 hr. From gel slices numbers 19 and 20 a precipitin line was formed.

eluates from these two slices plus one slice on either side of these yielded inhibitory activity in the lymphocytotoxicity assay against anti-HLA-A2 and anti-\(\beta_2\)-microglobulin (Fig. 5). The eluates of the other slices showed no inhibitory activity. Using a standard double-antibody immunofluorescence technique, \(\beta_2\)-microglobulin was identified on washed intact platelets (Fig. 6).

DISCUSSION

A considerable amount of detailed structural knowledge has become available about the serologically determined HLA antigen on human lymphocytes grown in culture and H-2 antigens from lymphocytes obtained from the spleens of mice.\(^{10-14,35-37}\) These studies have not been expanded to other cells because of the difficulty in obtaining enough soluble antigen with which to work. The use of the platelet in part overcomes this problem by allowing the harvesting of large numbers of platelets from the same individual or individuals with the same serologically defined HLA antigens. Despite these advantages, the yield from platelets has been very low.\(^{23}\) This has in part been explained by the smaller size of the platelet and the relative paucity of HLA antigens on the platelet surface, as compared with the lymphocyte.\(^{19}\) The present studies show that, in fact, platelets are a good source of HLA antigens, in that \(6 \times 10^{11}\) platelets (representing approximately 6 g of packed platelets) yielded 0.26 mg of purified HLA antigens, whereas 50 g of cultured lymphocytes yielded 1.05 mg of purified HLA antigens.\(^{6}\) This is a markedly improved yield in comparison with earlier work suggesting that \(4 \times 10^{11}\) platelets gave 0.01 mg of pure HLA antigen.\(^{23}\) This improved yield was due in part to the use of isolated platelet membranes for papain treatment, which allowed a two-fold increase in total HLA inhibitory activity and an 11-fold increase in specific activity, as compared with whole platelets.
Fig. 6. Photomicrograph of immunofluorescence of anti-β2-microglobulin on human platelets. The purified human platelets were incubated with a 1:20 dilution of rabbit anti-β2-microglobulin, washed, and then incubated with a 1:80 dilution of swine antirabbit IgG conjugated with fluorescein-isothiocyanate isomer I. The immunofluorescence was photographed at 400× magnification using a Zeiss Photomicroscope III. Appropriate controls showed no fluorescence, including one in which rabbit IgG was substituted for anti-β2-microglobulin.

The increased specific activity of HLA antigens when isolated platelet membranes are used can be explained by the relative decrease in non-HLA-containing material released from inside the platelet with papain treatment. But it is not clear why the total yield of HLA inhibitory activity should be greater with isolated platelet membranes than with intact platelets. This may be due to contamination of the isolated platelet membranes with intracellular membranes. Platelet glycoproteins have been shown to be present in large quantities on intracellular membranes. Possibly these intracellular membranes also contain HLA antigens in sufficient quantities to increase the yield of papain-solubilized HLA antigens. Alternatively, the isolation of the platelet membranes may result in topographic alteration in the membrane, allowing a more extensive cleavage of the HLA antigens by papain.

By the use of techniques similar to those employed to purify H-2 and HLA antigens from lymphocytes, platelet HLA antigens were purified. The HLA antigenic material solubilized from platelets by papain has an estimated molecular weight by exclusion chromatography of 40,000 ± 3500 d. This is less than the 48,000-d piece obtained from lymphocytes by papain digestion. Figure 2 depicts the chromatogram obtained for HLA-A2 activity. Other HLA activities represented on the same platelets had slightly different peak activities in relation to HLA-A2 activity but always remained within the β2-microglobulin activity range. This is identical to the results previously reported using cultured human lymphocytes. On further purification of the HLA antigens on ion-exchange chromatography (Fig. 3) there was even more distinct separation of the HLA specificities, as has
also been shown with papain-soluble lymphocyte HLA antigens. These purification procedures resulted in a 540-fold increase in the specific activity of HLA-A2 antigen and a 135-fold increase in the specific activity of β2-microglobulin (Table 1).

Figures 2 and 3 show that β2-microglobulin co-chromatographs with the HLA antigenic activity. A small amount of β2-microglobulin inhibitory activity could be found in the low-molecular-weight range on G-100 chromatography if the effluent was concentrated. The studies with non-SDS-PAGE further showed the association of β2-microglobulin with HLA activity. On SDS-PAGE gels there appears a 12,000-d band (Fig. 4) similar to that seen with lymphocyte HLA antigens. The separation of β2-microglobulin from the 26,000-d piece on SDS-PAGE represents breakage of a noncovalent linkage between the two chains. Figure 5 shows the immunofluorescence pattern for β2-microglobulin on the platelet. Previous studies quantitating β2-microglobulin have shown it to be present primarily in the platelet membrane. The pattern of immunofluorescence shows its general distribution over the entire platelet surface. These data taken as a whole suggest a key interrelationship between the HLA antigen and β2-microglobulin. The lymphocyte HLA antigen does not need the β2-microglobulin for antigenic activity. Whether that is true in the platelet is unknown.

The SDS-PAGE gels of the DEAE-purified platelet HLA antigen show a high-molecular-weight band of 26,000 d thought to represent the HLA antigenic activity (Fig. 4, gel D). This band is lighter than the corresponding band obtained from lymphocyte HLA antigens, which is 34,000 d. This difference appears to be confirmed, as seen by the difference in the estimated molecular weight of the complete molecule on gel exclusion chromatography, i.e., 40,000 d for the platelet versus 48,000 d for the lymphocyte. Whether this difference in molecular weight is due to a basic structural variation or is related to the position on the polypeptide chain accessible to papain cleavage is unclear. In view of the surface difference between the platelet and lymphocyte, there may be a difference in the attachment of HLA antigens into the membrane. But Bernier and associates have suggested that the HLA platelet antigens solubilized in NP-40 are composed of a 90,000-d piece plus a 45,000-d piece as determined on SDS-PAGE, whereas the estimate for the lymphocyte is 44,000 d. The techniques used in these studies were different, and the molecular weight estimates are not entirely comparable.

Our studies show for the first time on a normal human nonlymphocyte noncultured cell that the basic structure of the HLA antigen is similar to that described for the lymphocyte. The platelet HLA antigen is composed of a 12,000-d piece immunologically identical with β2-microglobulin and a 26,000-d piece similar to the 34,000-d heavy chain of the lymphocyte. It is not clear from our data if the association of these two pieces occurs in the platelet membrane. But in view of the data from lymphocytes and other malignant human cells that show partial co-capping of HLA antigens and β2-microglobulin, a similar association in the platelet membrane could be expected to occur.

The question why platelets possess HLA antigens is not clear. Antibodies directed to specific HLA antigens on the platelet surface can cause platelet aggregation. Thus it appears that alterations of the HLA antigen or structures around this antigen result in platelet activation. The surface determinants triggering this activation are of great interest in understanding and modifying platelet
activation. Further evaluation of platelet HLA antigens should yield a clearer picture of the similarities and differences of various cells and should yield some clues to the functional role of HLA antigens.

The main problem in working with nonlymphoid cells is the small quantity of antigen that can be isolated. The platelet yields less HLA antigen per cell than the lymphocyte, and it cannot be grown in tissue culture. Yet large quantities of normal platelets can be harvested with relative ease from the same donor or donors with identical HLA antigens, thus overcoming some of the quantity limitations.

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