Isolation and Characterization of the Receptor on Human Neutrophils That Mediates Cellular Adherence*

(Received for publication, December 22, 1986)

Dennis D. Hickstein‡, Juris Ozols§, Stuart A. Williams, Jacques U. Baenziger¶, Richard M. Locksley¶¶, and Gerald J. Roth

From the Departments of Medicine, Seattle Veterans Administration Medical Center and the University of Washington, Seattle, Washington 98108; the Department of Biochemistry, University of Connecticut Health Center, Farmington, Connecticut 06032; and the Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110.

The receptor on human neutrophils (polymorphonuclear leukocytes or PMN) that mediates cellular adherence has been purified from the peripheral blood PMN obtained from an individual with chronic myelogenous leukemia (CML). This receptor consists of two noncovalently associated subunits, designated αM (Mac-1α, CD11b) (M, = 170,000) and β (Mac-1β, CD18) (M, = 100,000), respectively, which are identical on normal and CML PMN. The subunits were purified by monoclonal antibody 60.1-Sepharose (anti-αM) affinity chromatography and separated in 5-nmol quantities by high pressure liquid chromatography on a TSK-4000 gel filtration column. Subunits were characterized by amino acid composition, N-terminal amino acid sequence, and carbohydrate content. The N-terminal sequence of the human PMN αM subunit contains regions of homology with the human platelet glycoprotein IIbα. We conclude that nanomole amounts of individual αM and β subunits of the receptor on human PMN that mediates cellular adherence can be isolated and separated using CML PMN.

To perform its role in host defense the human neutrophil (polymorphonuclear leukocyte or PMN) must adhere to the endothelium, migrate to the site of infection, and ingest appropriately opsonized particles. Recently, a PMN surface membrane antigen that is centrally involved in these adherence-related activities has been described (1–4). This glycoprotein complex is composed of dimeric subunits, designated αM (Mac-1α, Mola, CD11b) and β (Mac-1β, Molβ, CD18) (1–4).

The αM:β adherence complex exists as a member of a family of related leukocyte adherence glycoproteins present on the cell surface of different populations of leukocytes in both mouse and man (1, 5). Members of this family consist of distinct α subunits designated: αL (LFA-1α, CD11a) (1, 4), αM (Mac-1α, Mola, CD11b) (2, 4), and α (p150, CD11c) (1, 4), noncovalently associated with an identical β subunit (β subunit of LFA-1α, Mac-1α, p150, CD18) (1–4) in an αβ, dimeric structure (1). The approximate molecular masses for αL, αM, αX, and β are 180, 170, 150, and 100 kDa, respectively (1–8). The αM:β complex is the predominant member on PMN and monocytes, whereas the αL:β is the major member of the family on T-lymphocytes (1–8). Adherence of T-lymphocytes appears to be mediated by the αL:β antigen complex (8).

Two observations indicate that the αM:β complex plays a critical role in PMN physiology. First, monoclonal antibodies (mAb) directed against the subunits of this complex block PMN adherence-related activities (1–8). Second, the PMN from children whose leukocytes are severely deficient in the member subunits of this glycoprotein family display profound defects in PMN adherence and adherence-related functions, including phagocytosis, in vitro assays (3, 9, 10). Clinically, these children suffer from recurrent and severe bacterial infections that frequently culminate in death (3, 9, 10).

Two members of this leukocyte family, αL (LFA-1) and αM (Mac-1), have been isolated from the murine cell lines EL-4 and P388D1, respectively (11). Sequence analysis indicated that the terminal 19 residues of the two subunits contained 33% homology (11). Thus, the genes for these proteins might have risen by gene duplication from a primordial gene. The N-terminal amino acid sequence of the human platelet adherence glycoprotein IIbα was demonstrated recently to contain regions of similarity to both murine aL and murine αM (12). Preliminary molecular characterization of these antigens, obtained by transfection of L-cells, suggested that the genes for αL and αM and platelet glycoprotein IIb/IIIa were located within a 20-kilobase segment of human DNA (13).

Purification of nanomole amounts of the PMN adherence glycoproteins αM and β for structural-functional relationships has proven difficult. In this report we describe the purification of nanomole amounts of both subunits from the peripheral blood PMN of an individual with chronic myelogenous leukemia (CML). Purification was performed with a one-step method using mAb 60.1-Sepharose (anti-αM) affinity chromatography. The individual subunits were separated with a TSK-4000 gel filtration column using high pressure liquid chromatography (HPLC). Sequence analysis indicated that the murine and human αM subunits are homologous and...
that the human αM subunit is related to human platelet glycoprotein Ibα.

**MATERIALS AND METHODS**

**Monoclonal Antibodies—**mAb 60.1 (IgG1, κ) binds to the 170-kDa αM subunit (6), while mAb 60.3 (IgG3, κ) binds to the 100-kDa β subunit (3, 6, 7). mAbs were conjugated to CNBr-activated Sepharose at approximately 5 mg of antibody/ml of gel according to the manufacturer’s directions (Pharmacia P-L Biochemicals).

**Gel Electrophoresis—**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (14).

**Cell Preparation—**Neutrophils (PMN) were obtained from the peripheral blood of normal individuals and two individuals with chronic myelogenous leukemia (CML; chronic phase) (15) according to standard procedures (16). HL-60 promyelocytic leukemia cells (provided by Dr. Steven J. Collins, Fred Hutchinson Cancer Research Center, Seattle, WA) were grown in RPMI 1640 (Irving Scientific, Santa Ana, CA) supplemented with 10% heat-inactivated fetal bovine serum (HyClone Laboratories, Logan, UT) (17). HL-60 cells were quantitated using an Ortho 50H cytofluorograph interfaced to a model cytocentrifuge for the preparation of normal PMN, CML PMN, and HL-60 cells. Immunofluorescence of individual cells was conducted to determine a source of each subunit for large-scale purification, indicated that CML PMN were a potential source of both αM and β.

The αM and β subunits were immunoprecipitated from cell lysates of normal PMN, CML PMN, and HL-60 cells. To create the cell lysates, cells (10¹⁰) were solubilized by incubation at 4°C for 30 min in a cell lysis buffer consisting of 0.5% Nonidet P-40, 0.15 M NaCl, 0.05 M Tris, 2 mM phenylmethylsulfonyl fluoride (Sigma), 50 mM iodoacetamide (Sigma), 1 mM pepstatin (Sigma), and 1 mM diisopropyl fluorophosphate (Sigma) at pH 8.3. Nuclei were removed by centrifugation at 5000 × g at 4°C for 30 min. The supernatant was further clarified by centrifugation at 100,000 × g at 4°C for 60 min. The αM and β subunits were immunoprecipitated from the cell lysate by incubating the lysate with 0.1 ml of a 1:1 liquid-solid solution of mAb 60.1-Sepharose at 4°C for 60 min. The immunoprecipitates were collected by centrifugation and washed eight times with buffer containing 0.5% Nonide P-40, 0.45 M NaCl, 0.05 M Tris, 0.1% SDS, at pH 8.3. Prior to gel electrophoresis samples were reduced with 5% (v/v) 2-mercaptoethanol at 100°C for 5 min. Proteins were visualized by Coomassie Blue staining of the gels.

**Purification of the αM and β Subunits—**For purification of quantities of αM and β greater than 0.1 mg, the starting material consisted of 10–50 g of PMN obtained by leukapheresis of individuals with CML and leukocytosis (white blood cell count > 50,000/mm³). The supernatant (200–800 ml) from the final centrifugation at 100,000 × g was applied directly to a 2-ml mAb 60.1-Sepharose column at a flow rate of 15 ml/h. Following washing with cell lysis buffer at pH 8.0, the column was washed with 0.01 M Tris, 0.1% deoxycholate, 2 mM phenylmethylsulfonyl fluoride. The column eluate was 0.050 M triethylammonium, 0.1% deoxycholate, 2 mM phenylmethylsulfonyl fluoride, pH 11.5, at a flow rate of 15 ml/h. Preliminary studies, using a gradient of increasing pH, had demonstrated that the majority of αM eluted from mAb 60.1-Sepharose at pH 11.5 (19). Fractions of 1.8 ml were collected into 0.2 ml of 1 M Tris, pH 8.5. The protein content of fractions was estimated by measuring absorbance at 280 nm (A280) and fractions with an A280 ≥ 0.1 were pooled and dialyzed overnight at 4°C in 0.001 M Tris, 0.01% deoxycholate, pH 8.2. Following dialysis, the eluate was lyophilized.

The αM and β subunits were separated using HPLC. The dialyzed and lyophilized eluate from the mAb 60.1-Sepharose column was dissolved in 6 M guanidine containing 0.050 M dithiothreitol at 37°C for 4 h and alkylated by incubation with iodoacetic acid for 25 min. The αM and β subunits were dialyzed, lyophilized, redissolved in 2% SDS to achieve a final concentration of 1 mg of protein/ml of 2% SDS, and injected onto a TSK-4000 preparative (2.1 × 60 cm) gel filtration column at a flow rate of 2 ml/min. Eluted proteins were detected by absorbance at 280 nm. Fractions encompassing the peak for each subunit were lyophilized, and the proteins precipitated by PAGE followed by Coomassie Blue and silver staining. Fractions of αM and β collected from the initial HPLC were subjected to a repeat HPLC separation procedure.

The amino acid compositions of the peptides were determined on acid hydrolysates of the samples using an updated single column Beckman 120C automatic amino acid analyzer (20). NH₂-terminal amino acid sequence of the αM and β subunits was determined by automated Edman degradation with the use of an Applied Biosystems (Foster City, CA) model 470A gas-phase sequenator (21). Neutral and amino sugars were determined by gas chromatography as described (21). Analyses were performed with a Varian 3700 gas chromatograph with an OV 101 WCOT column and flame ionization detector using the on-column technique of Grob and Grob (22).

**RESULTS**

Expression of PMN Adherence Complex on Human PMN, CML PMN, and HL-60 Promyelocytic Leukemia Cells—In initial studies we analyzed the expression of the αM and β subunits on the peripheral blood PMN from normal individuals, the PMN from two individuals with CML, and myeloid cells of the HL-60 human promyelocytic leukemia cell line. Subunit expression was quantitated using subunit-specific mAb and indirect immunofluorescence followed by flow cytometry. The mean fluorescence of each subunit was nearly identical on normal human PMN and CML PMN (Table I). The αM subunit was not expressed on undifferentiated HL-60 cells, but it was expressed following differentiation with Me₂SO (23). However, the surface density of αM on Me₂SO-treated HL-60 cells never reached the levels present on normal or CML PMN (Table I). These experiments, which were conducted to determine a source of each subunit for large-scale purification, indicated that CML PMN were a potential source of both αM and β.

mAb 60.1-Sepharose (anti-αM) was used to immunoprecipitate the αM and the noncovalently associated β subunit from normal and CML PMN. The subunits precipitated from both normal PMN and CML PMN had identical αM and β subunit content of both αM and β on normal PMN, CML PMN, and HL-60 cells. The absence of structural heterogeneity of these subunits in human neutrophils that mediates cellular adherence

**TABLE I**

| Subunit        | Relative mean fluorescence |
|----------------|--------------------------|
| αM             | β                         |
| Normal PMN     |                          |
| (n = 2)        | 131 ± 1.0                 | 137 ± 2.0                  |
| CML PMN        | 129.5 ± 3.5               | 136.5 ± 4.5                |
| (n = 2)        |                          |                            |
| Undifferentiated| 55.5 ± 5.3                | 113.7 ± 3.9                |
| HL-60 (n = 3)  |                          |                            |
| Me₂SO-differentiated| 85.5 ± 6.0            | 121.0 ± 4.1                |
| HL-60 (n = 3)  |                          |                            |

* Results were analyzed by flow cytometry and expressed as the relative mean fluorescence on an arbitrary log scale from 0 to 200. Background fluorescence using an irrelevant first antibody and the fluorescein isothiocyanate-linked sheep anti-mouse antibody. Values shown represent the mean ± S.E.

mAb 60.1 detected αM (6) and mAb 60.3 detected β (7).

n indicates the number of experiments.
ease in obtaining large numbers of PMN using leukapheresis of individuals with CML (15).

Purification and Separation of the αM and β Subunits—
mAb 60.1-Sepharose (anti-αM) affinity chromatography was utilized to purify both subunits from a cell lysate created from 25 g of CML PMN (“Materials and Methods”). Localization of the αM and β subunits to discrete fractions of the eluate is shown (Fig. 1). Proteins eluted from the mAb 60.1-Sepharose column were injected onto a preparative TSK-4000 HPLC gel filtration column and five discrete peaks were recorded by measurement of A280 (Fig. 2). The αM and β subunits eluted in peaks designated A and B, respectively (Figs. 2 and 3). Coomassie Blue staining of an overloaded gel (Fig. 3, overload) as well as silver staining of an SDS-PAGE gel (data not shown) both demonstrated that αM (170 kDa) contained approximately 5% of β (120 kDa), and vice versa. The fractions containing the αM and β subunits in the initial HPLC gel filtration were each subjected to repeat HPLC gel filtration to further purify each subunit (Fig. 2, panels A and B). The protein peaks collected from the second HPLC run were used to determine amino acid composition, NH2-terminal amino acid sequence, and carbohydrate content.

Amino acid compositions were determined for each subunit. The number of residues for αM was estimated from the Mr of the αM glycoprotein (170,000) and the αM protein (130,000) (24). Similarly, the number of residues for β was estimated from the Mr of the β glycoprotein (100,000) and the β protein (72,000) (24). Using this data the number of residues for αM was 1169 and the number of residues for β was 639. The absorption coefficient E was estimated from the absorbance of purified protein in solution and the amino acid content; for the αM subunit, E = 5.49 and for β E = 8.70 (25).

Results of the amino acid composition of αM and β indicated that the proteins were similar to each other (Table II). The remarkable difference lies in the increased cysteine content in β compared to αM. Tais high cysteine content of β is consistent with the observation that the higher Mr of this protein on SDS-PAGE after reduction is due to the presence of intra-chain disulfide bonds.

To define these two proteins more precisely, sequence analysis was performed. Comparison of 13 NH2-terminal amino acid residues of the human αM subunit with the murine αM (Mac-1) (11) indicated only two substitutions (Table III). In the human sequence Asn replaced His at position 7 and Ala replaced Pro at position 8, although some ambiguity may exist in assigning Pro at position 8 of the murine protein (11). In addition, human PMN αM contained approximately 40% sequence homology with another hematopoietic cell adherence glycoprotein, that of platelet glycoprotein IIbα (12). No amino acid sequence could be obtained from 2 nmol of PMN β subunit, suggesting the presence of a blocked NH2 terminus.

The carbohydrate composition for αM and β, respectively, were: fucose 1.5 and 1.2, mannose 16.5 and 3.1, galactose 8.4 and 2.6, and N-acetylglucosamine, 7.1 and 13.6, expressed as nanomole of carbohydrate/nanomole of protein. Sialic acid residues were present in both subunits but were not quantitated.

Fig. 1. Large-scale purification of αM/β from PMN from an individual with CML. Twenty-five grams of peripheral blood PMN from an individual with CML were obtained by leukapheresis, lysed in the presence of multiple protease inhibitors, and isolated using mAb 60.1-Sepharose affinity chromatography. The mAb-Sepharose column was eluted at pH 11.5 and the eluate was collected in 2-ml fractions. The protein in fractions 1–5 was acetone precipitated at −20 °C, reduced, analyzed on 7% SDS-PAGE, and stained with Coomassie Blue.
chronic phase demonstrated that the αM and population capable of serving as a source of antigen for large-phase (15) were demonstrated to express the αM and identical as assessed by one-dimensional SDS-PAGE. Individually with CML/chronic phase provided an excellent source of starting material for antigen preparation since: 1) they maintain peripheral white blood cell counts 5–20 times greater than normal individuals; 2) their cells are primarily mature PMN; and 3) they are generally in otherwise good health, thus tolerating leukapheresis for removal of white blood cells (15).

Amino acid composition of human PMN αM and β subunits

| Amino acid | αM (residue/peptide mol/100 mol of amino acid) | β (residue/peptide mol/100 mol of amino acid) |
|------------|-----------------------------------------------|-----------------------------------------------|
| Lysine     | 45.6                                          | 28.5                                          |
| Histidine  | 20.9                                          | 17.9                                          |
| Arginine   | 72.6                                          | 38.0                                          |
| Cysteine   | 28.0                                          | 46.7                                          |
| Asparagine | 113.5                                         | 66.8                                          |
| Threonine  | 74.7                                          | 34.5                                          |
| Serine     | 95.2                                          | 41.2                                          |
| Glutamate  | 133.0                                         | 59.9                                          |
| Aspartate  | 56.3                                          | 55.9                                          |
| Alanine    | 66.7                                          | 32.9                                          |
| Valine     | 93.4                                          | 37.5                                          |
| Methionine | 3.5                                           | 1.3                                           |
| Isoleucine | 45.8                                          | 4.3                                           |
| Leucine    | 135.6                                         | 67.4                                          |
| Tyrosine   | 31.8                                          | 19.8                                          |
| Phenylalanine | 61.8                                   | 20.4                                          |
| Tryptophan | 6 (6.5)                                       | 7.8                                           |

* Detected as S-carboxymethylcysteine.
* Tryptophan content was estimated by absorbance (25).

Micro sequence analysis of human PMN αM, murine αM, and human platelet Ibα

| Position | Human αM | Mouse αM | Human platelet Ibα |
|----------|----------|----------|---------------------|
| 1        | Phe      | Phe      | Leu                 |
| 2        | Asn      | Asn      | Asn                 |
| 3        | Leu      | Leu      | Leu                 |
| 4        | Asp      | Asp      | Asp                 |
| 5        | Thr      | Thr      | Pro                 |
| 6        | Glu      | Glu      | Val                 |
| 7        | Asn      | His      | Glu                 |
| 8        | Ala      | Pro      | Leu                 |
| 9        | Met      | Met      | Thr                 |
| 10       | Thr      | Thr      | Phe                 |
| 11       | Phe      | Phe      | Tyr                 |
| 12       | Gln      | Gln      | Ala                 |
| 13       | Glu      | Glu      | Gly                 |

* Ref. 11.
* Ref. 12.

DISCUSSION

We purified and separated the αM and β subunits of the receptor on human PMN that mediates cellular adherence using a cell lysate created from the peripheral blood PMN of an individual with CML. The initial isolation utilized mAb 60.1-Sepharose (anti-αM) affinity chromatography. Separation of the subunits required HPLC using a TSK-4000 gel filtration column. The subunits were characterized by amino acid composition, amino acid sequence, and carbohydrate content.

Initial studies were designed to identify a cell line or cell population capable of serving as a source of antigen for large-scale purification. PMN from individuals with CML/chronic phase (15) were demonstrated to express the αM and β subunits in amounts identical to those present on normal PMN. Our experiments using immunoprecipitations from PMN obtained from normal individuals and those with CML/chronic phase demonstrated that the αM and β subunits were identical as assessed by one-dimensional SDS-PAGE. Individuals with CML/chronic phase provided an excellent source of starting material for antigen preparation since: 1) they

REFERENCES

1. Sanchez-Madrid, F., Nagy, J. A., Robbins, E., Simon, P., and Springer, T. A. (1983) J. Exp. Med. 158, 1795–1795
2. Arnaout, M. A., Todd, R. F., III, Dana, N., Melamed, J., Schlossman, S. F., and Colten, H. R. (1983) J. Clin. Invest. 72, 171–179
3. Beatty, P. G., Harlan, J. M., Ochs, H. D., Price, T. H., Rosen, H., Taylor, R. F., Hansen, J. A., and Klebanoff, S. J. (1984) Lancet 1, 535–537
4. McMichael, A. (1987) Joint Report of the Third International Workshop on Human Leukocyte Differentiation Antigens. Oxford Press, Oxford, in press
5. Trowbridge, I. S., and Omary, M. D. (1981) J. Exp. Med. 154, 1517–1523
6. Wallis, W. J., Hickstein, D. D., Schwartz, B. R., June, C. P., Ochs, H. D., Beatty, P. G., Klebanoff, S. J., and Harlan, J. M. (1986) Blood 67, 1097–1115
7. Hickstein, D. D., Locksley, R. M., Beatty, P. G., Smith, A., Stone, D. M., and Root, R. K. (1986) Blood 67, 1054–1062
8. Springer, T. A., Davignon, D., Ho, M. K., Kurzinger, K., Marts, E., and Sanchez-Madrid, F. (1982) Immunol. Rev. 68, 111–135
9. Arnaout, M. A., Pitt, J., Cohen, H. J., Melamed, J., Rosen, F. S., and Colten, H. R. (1982) N. Engl. J. Med. 306, 683–690
10. Springer, T. A., Thompson, W. S., Miller, L. J., Schmalsteig, F. C., and Anderson, D. C. (1984) J. Exp. Med. 160, 1901–1918
Neutrophil Receptor Mediating Cellular Adherence

11. Springer, T. A., Teplow, D. B., and Dreyer, W. J. (1985) *Nature* **314**, 540-542
12. Charo, I. F., Fitzgerald, L. A., Steiner, B., Rall, S. C., Bekeart, L. S., and Phillips, D. R. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 8351-8355
13. Cosgrove, L. J., Sandrin, M. S., Rajasekariah, P., and McKenzie, I. F. C. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 752-756
14. Laemmli, U. K. (1970) *Nature* **227**, 680-685
15. Rundles, R. W. (1987) in *Hematology* (Williams, W. J., Beutler, E., Ersliev, A. J., and Lichtman, M. A., eds) Vol. 1, pp. 196-213, McGraw-Hill, New York
16. Boyum, A. (1968) *Scand. J. Clin. Lab. Invest.* **21**, (suppl.) **97**, 77-89
17. Collins, S. J., Gallo, R. C., and Gallagher, R. E. (1977) *Nature* **270**, 347-349
18. Collins, S. J., Ruscetti, F. W., Gallagher, R. E., and Gallo, R. C. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 2468-2462
19. Kürzinger, K., and Springer, T. A. (1982) *J. Biol. Chem.* **257**, 12412-12418
20. Ozols, J. (1986) *J. Biol. Chem.* **261**, 3965-3979
21. Bäenziger, J., Kornfeld, S., and Kochwa, S. (1974) *J. Biol. Chem.* **249**, 1889-1896
22. Grob, K., and Grob, K., Jr. (1978) *J. Chromatogr.* **151**, 311-320
23. Hickstein, D. D., Smith, A. J., Fisher, W., Beatty, P. G., Schwarz, B. R., Harlan, J. M., Root, R. K., and Locksley, R. M. (1987) *J. Immunol.* **138**, 513-519
24. Sastre, L., Kishimoto, T. K., Gee, C., Roberts, T., and Springer, T. A. (1986) *J. Immunol.* **137**, 1060-1065
25. Fruton, J. S., and Simmonds, S. (1958) *General Biochemistry*, 2nd Ed, pp. 72-73, John Wiley and Sons, New York