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Research Article

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Deficiency of a Surface Membrane Glycoprotein (Mo1) in Man

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Abstract. Deficiency of a granulocyte surface glycoprotein of 150,000-D had been associated with defective C3- and IgG-dependent phagocytosis in a patient with recurrent bacterial infections. By using monoclonal antibodies, we found that this patient's granulocytes, monocytes, and null cells were deficient in Mo1 (equivalent to OKM1 and Mac-1), a cell surface molecule consisting of two noncovalently linked glycoproteins of 155,000 and 94,000 D. The 155,000-D subunit is closely associated with the human complement receptor that recognizes C3bi and/or a further degradation product termed C3dg (C3bi receptor); the 94,000-D subunit has been shown to be shared, on normal cells, by two other surface membrane glycoproteins: lymphocyte function-associated antigen-1 (LFA-1) and P-150,95. Both subunits of Mo1 were deficient on the patient's granulocytes as determined by immunoprecipitation with subunit-specific monoclonal antibodies as well as fluorescence analysis. Mo1-deficient monocytes, like granulocytes, had defective C3- and IgG-dependent phagocytosis. Natural killing activity by the patient's peripheral blood leukocytes was normal. Mo1-deficient granulocytes and monocytes rosetted normally with sheep erythrocytes coated with C3bi. This rosetting was totally inhibited by a mixture of anti-Mo1 and anti-C3b (the major fragment of C3) receptor antibodies but not by either antibody alone. Since monoclonal antibodies to the 155,000-D subunit of Mo1 can inhibit C3bi receptor binding, immune phagocytosis, opsonized zymosan-induced degranulation, and superoxide generation by normal phagocytes (functions which are defective in Mo1-deficient cells), it appears likely that Mo1 deficiency may in part underlie the functional aberrations leading to recurrent bacterial infections in man.

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Introduction

Mo1 is a cell surface glycoprotein present on human granulocytes, monocytes, and null cells (1). It consists of two noncovalently linked proteins of 155,000 and 94,000 molecular masses (2). Monoclonal antibodies to this antigen and to a similar surface antigen in the mouse and human (Mac-1) specifically inhibit complement C3bi-dependent rosette formation (3, 4). In addition, anti-Mo1 blocks phagocytosis by human granulocytes of C3- or IgG-opsonized particles as well as lysosomal enzyme release and superoxide generation by granulocytes in response to opsonized zymosan (3) (Arnaout, M. A., and J. Melamed, unpublished observations). Impaired phagocytosis and opsonized zymosan-induced degranulation and superoxide production were previously noted in granulocytes from a patient with recurrent bacterial infections (5). The patient's cells were found to be deficient in a surface glycoprotein of molecular mass of ~150,000 D (5). The similarities in the functional defects found in this patient and those produced in normal cells by anti-Mo1 led us to look for Mo1 on the patient's cells to gain more insight into the molecular mechanisms that led to the cellular functional aberrations detected in this patient. We found that: (*a*) The patient's granulocytes, monocytes, and null cells were deficient in Mo1. (*b*) Mo1-deficient monocytes had impaired ability to ingest C3- or IgG-coated particles. (*c*) Rosette formation between the patient's phagocytes and sheep eryth-

rocytes coated with C3bi[1] (a fragment of C3b generated upon cleavage of surface-bound C3b by factors I and H) was totally inhibited by a mixture of anti-Mo1 and a polyclonal antibody to the receptor for the major cleavage product of C3 (C3b), but not by either antibody alone. (*d*) Peripheral blood leukocytes (PBL) deficient in Mo1 had normal natural killing (NK) activity.

Methods

Cell separation. Human neutrophils were prepared from EDTA anticoagulated venous blood by Ficoll-Hypaque centrifugation followed by hypotonic lysis of the erythrocyte pellet (6). Monocytes were isolated from the interphase mononuclear cell fraction by adherence to plastic petri dishes (1). A null cell-enriched population was isolated from mononuclear cells by binding of complement fixing monoclonal antibodies to T12 (7), I-2 (8) and Mo2 (1) surface markers (at 1:100 dilutions each, for 30 min at 4°C), followed by complement lysis (rabbit complement, Pel-Freeze Biologicals, Rogers, AR) for 60 min at 37°C. These steps depleted T and B lymphocytes and monocytes. The remaining cells were enriched for Mo1-positive and Mo2-negative null cells.

Monoclonal antibodies and heteroantisera. Monoclonal antibodies to Mo1, Mo2, Mo5, and Mac-1 were produced as previously described (9, 10). The OKM1O monoclonal antibody was obtained from Ortho Pharmaceutical (Raritan, NJ). Two additional monoclonal antibodies to Mo1 were kindly provided by Dr. J. Griffin, of the Dana Farber Cancer Institute. The $F(ab')_2$ fragment of IgG2a anti-Mo1 and anti-Mo5 were generated by pepsin digestion followed by gel filtration (11). The TS1/18 monoclonal antibody to the 94,000-D subunit of lymphocyte function-associated antigen-1 (LFA-1) and Mo1/Mac-1/OKM1 was produced as described (12). A $F(ab')_2$ fragment of rabbit IgG directed against the human C3b receptor (anti-CR1), was a generous gift of Dr. D. Fearon, Brigham and Women's Hospital, Boston, MA (13).

Immunoprecipitation. Intact neutrophils were treated with diisopropylfluorophosphate and then ^{125}I-surface labeled with Iodogen (Pierce Chemical Co., Rockford, IL) as previously described (5). ^{125}I-incorporation was comparable in patient and control cells (data not shown). After labeling, cells were solubilized in 0.5% Non-Idet P40 (NP40) in phosphate-buffered saline (PBS) and the lysates centrifuged at 105,000 *g* for 30 min. The supernatants were precleared with immune complexes consisting of a control mouse monoclonal antibody, rabbit IgG anti-mouse Ig, and heat-inactivated formalin-fixed *Staphylococcus aureus.* Precleared lysates were then incubated for 12–16 h with the specific monoclonal antibody. Rabbit antiserum to mouse Ig or a rat, anti-mouse kappa chain monoclonal antibody was added and incubation continued for 1 h at 4°C, followed by *S. aureus* (30 min, 4°C). The insolubilized immune complexes were washed four times with PBS containing 2 mM phenyl-methyl-sulfonyl-fluoride and 0.1% Non-Idet P40 (NP40), and then extracted by boiling in Tris buffer, pH = 6.8 (sample buffer), containing 2% sodium dodecyl sulfate and 5% 2-mercaptoethanol. Eluates were electrophoresed on polyacrylamide gels as described (14).

Immunofluorescence studies. Indirect immunofluorescence of intact cells or cells permeabilized with methanol (to detect intracellular antigen) (15) was done by using the monoclonal antibodies and fluorescein-conjugated, goat anti-mouse IgG and IgM as previously described (1). Staining patterns of cell populations were generated by using a FACS-1 cytofluorograph (Becton-Dickinson & Co., Westwood, MA).

Complement or IgG-coated particles. Sheep erythrocytes (E) in Alsever's solution were obtained from M. A. Bioproducts (Walkersville, MD). IgG rabbit antibody directed to E was purchased from Cordis Laboratories (Miami, FL) and serum made by immunizing rabbits with E stroma (16) was used as a source of IgM antibody to E.

Sheep erythrocytes coated with rabbit IgG anti-E (EIgG) were made by incubating E with the highest, nonagglutinating concentration of antibody in PBS for 15 min at 37°C. Cells were then washed several times in PBS.

Sheep erythrocytes coated with C3b (EAC43b) were made by first incubating E with rabbit anti-E IgM antibody. This was followed by sequential incubation with guinea pig C1, human C4, guinea pig C2, and then human C3 as described by Rapp and Borsos (16). C1 and C2 were allowed to dissociate by 2 h-incubation at 37°C in veronal-buffered saline containing 10 mM EDTA.

C3bi-coated sheep erythrocytes (EAC43bi) were made by incubating EAC43b with purified factor I (10 μg/ml) and factor H (50 μg/ml) (17) in Hanks' balanced salt solution containing Ca (1.27 mM) and Mg (1.23 mM) (HBSS) for 1 h at 37°C as described (3). Alternatively, EAC43bi were generated by incubating EAC43b in whole plasma that was extensively preabsorbed with antibody-coated sheep erythrocytes at 0°C. 1.5×10^8 EAC43b cells were incubated with 200 μl of preabsorbed human EDTA plasma at 1:10 dilution (as a source of factors H and I) for 30 min at 37°C followed by several washes in HBSS containing 1 mg/ml soybean trypsin inhibitor (Worthington). EAC43bi did not bind to human erythrocytes (18) but rosetted with human granulocytes and monocytes. Rosetting of EAC43bi with normal phagocytes was inhibited by monoclonal antibodies to Mo1 but not by a rabbit antibody to the human C3b receptor (anti-CR1).

C3d-coated sheep erythrocytes (EAC43d) were obtained by incubating EAC43bi with TPCK-trypsin (Millipore Co., Freehold, NJ) at 0.1 μg/ml in PBS for 45 min at 37°C (13). EAC43d cells did not rosette with human neutrophils but rosetted strongly with Raji cells.

Serum-opsonized lipopolysaccharide-coated oil red O (ORO) particles were prepared as previously described (19).

Rosetting. Granulocytes and monocytes were kept at 4×10^6/ml in HBSS, 2 mg/ml bovine serum albumin, and 1 mg/ml soybean trypsin inhibitor (Millipore Corp.). 25 μl of cells were incubated with 3 μg $F(ab')_2$ anti-Mo1, anti-Mo5, or 2 μg of $F(ab')_2$ anti-CR1 for 20 min at room temperature, after which 15 μl of indicator E at 1.5×10^8/ml were added and the mixture incubated for 40 min at 37°C. To assess rosette formation, the cells were gently resuspended and examined using a hemocytometer. Binding of three or more E to a leukocyte was considered a rosette.

Phagocytosis. One million monocytes suspended in 100 μl of RPMI-1640 were incubated with or without 5 μg of $F(ab')_2$ anti-Mo1 or control monoclonal antibody for 20 min at room temperature. 50 μl of EIgG at 2×10^8/ml were then added and the mixture incubated at 37°C for 1 h. Noningested E were hypotonically lysed with 0.0375 M sodium chloride for 5 s, after which isotonicity was established with 3M sodium chloride (20). Wright-stained cells were then counted to determine the percentage of cells that ingested one or more E.

Phagocytosis of C3-opsonized ORO particles by patient or control monocytes was performed as previously described (19).

NK assay. The ability of the patient's PBL to effect NK of the target cell K562 was compared with normal control PBL. K562 cells

1. *Abbreviations used in this paper:* C3b, the major fragment of C3; C3bi, a fragment of C3b generated upon cleavage of surface-bound C3b by factors I and H; C3bi receptor, a complement receptor that recognizes C3bi and/or a further degradation product termed C3dg; C3d receptor, a receptor specific for the C3d fragment of C3; CR1, the C3b receptor; E, sheep erythrocytes; EAC43b, sheep erythrocytes coated with C3b; EAC43bi, C3bi-coated sheep erythrocytes; EAC43d, C3d-coated sheep erythrocytes; EIgG, sheep erythrocytes coated with rabbit IgG anti-E; NK, natural killing, ORO, oil red O.

were labeled with chromium 51 and incubated with PBL at various effector to target cell ratios for 4 h at 37°C (21). Target killing was determined by 51 chromium release into the media. Percent lysis was calculated as previously reported (21).

Results

Immunoprecipitation of granulocyte membrane proteins by monoclonal antibodies. A monoclonal antibody to Mo1 immunoprecipitated from ^{125}I-surface-labeled normal granulocyte lysates, two noncovalently linked glycoproteins of 155 and 94 kD (Fig. 1 *A*). By using this same antibody, no bands were immunoprecipitated from the patient's ^{125}I-labeled granuloctyes (Fig. 1 *A*). This monoclonal anti-Mo1 reacts only with the 155-kD glycoprotein (3). To determine if the patient's cells were also missing the 94-kD subunit, a monoclonal antibody reactive with the 94-kD subunit of Mo1/OKM1 was used. This anti-94-kD antibody immunoprecipitated from normal cells the two subunits of Mo1 as well as two other surface proteins which share the same 94-kD subunit with OKM1/Mo1, namely LFA-1 (subunits of 177 and 95 kD) and P-150,95 (subunits of 150 and 95 kD) (Fig. 1 *B*, lane *f*) (22). No radiolabeled bands were seen with the patient's cells (Fig. 1 *B*, lane *c*). These data demonstrate that the surface of the patient's granulocytes is deficient in both the 155 and 94 kD polypeptides of Mo1 antigen as determined by subunit-specific monoclonal antibodies. To determine if the patient's neutrophils totally lack Mo1, a more sensitive radioimmunoassay was used (3). Increasing amounts of ^{125}I-Fab′ anti-Mo1 were incubated with the patient's granulocytes in the absence or presence of unlabeled Fab′ anti-Mo1 (100-fold molar excess). Scatchard analysis of the binding data revealed that the patient has 7,000±3,000 Mo1 sites/cell (mean±SD) vs 65,000±7,000 (mean±SD) for granulocytes from nine random individuals. These data indicate that the patient is deficient but not totally lacking the 155-kD subunit of Mo1.

Analysis of cell surface antigens by immunofluorescence. Fluorescence analysis confirmed that the patient's granulocytes were deficient in Mo1, but expressed normal amounts of other leukocyte markers including Mo5, J5, and beta$_2$-microglobulin (data not shown). The patient's granulocytes were also deficient in the equivalent Mac-1 antigen (data not shown). The patient's monocytes and null cells were also deficient in Mo1 when studied by indirect immunofluorescence technique. As can be seen in Fig. 2, immunofluorescent staining with anti-Mo2 (a monocyte-specific surface marker) of both the patient's and control monocytes were comparable; yet, only the control monocytes had detectable staining with anti-Mo1. Similarly, no anti-Mo1 staining was detected on the patient's null cell population. This population was defined by selecting the mononuclear cells which were T12-, Ia-, and Mo2-negative as described in Methods. A similar null cell population, which was isolated in parallel from normal control blood, had detectable immunofluorescent staining with the anti-Mo1 antibody (Fig. 2 *B*).

The possibility that the patient's cells had normal amounts of an altered Mo1 protein deficient in only one epitope was rendered unlikely by the use of independent monoclonal antibodies. Five separate monoclonal antibodies reactive with the Mo1 glycoprotein on normal cells failed to bind to patient's granulocytes as determined by indirect immunofluorescence analysis (data not shown).

One reason for the surface deficiency of Mo1 on the patient's cells could be that the antigen is sequestered in an intracellular

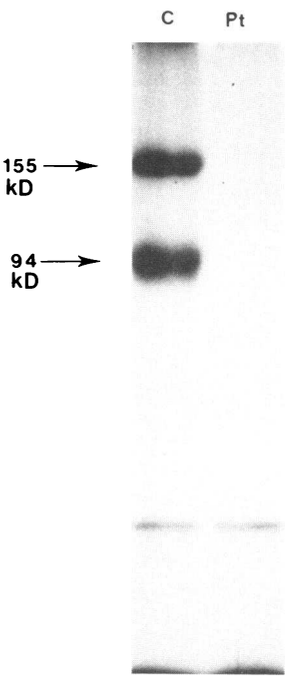

Figure 1 A. Radioautograph of a 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis of anti-Mo1 immunoprecipitates from ^{125}I-labeled granulocyte lysates from control (*left* lane) and patient (*right* lane).

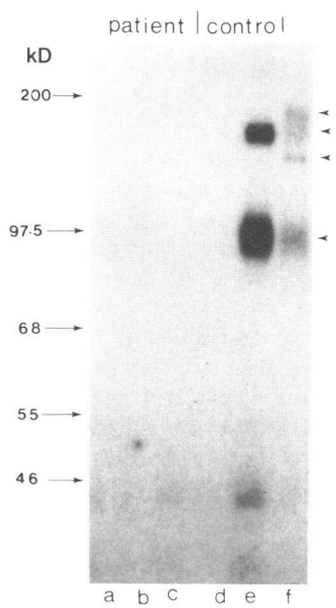

Figure 1 B. Radioautography of a 7% sodium dodecyl sulfate-polyacrylamide gel electrophoresis of monoclonal antibody immunoprecipitates from ^{125}I-surface labeled granulocyte lysates. No bands were immunoprecipitated from patient lysates with OKM10 (another monoclonal antibody to Mo1) (lane *b*), TS1/18 monoclonal antibody to the 94-kD subunit (lane *c*), or a control mouse Ig (lane *a*). Control cells had Mo1 (lane *e*). In addition to the 155-kD subunit of Mo1, monoclonal antibody to the 94 kD immunoprecipitated from normal cells two proteins of approximate molecular mass of 177 and 150 kD (arrow heads) (lane *f*). No bands were precipitated by a control mouse Ig (lane *d*).

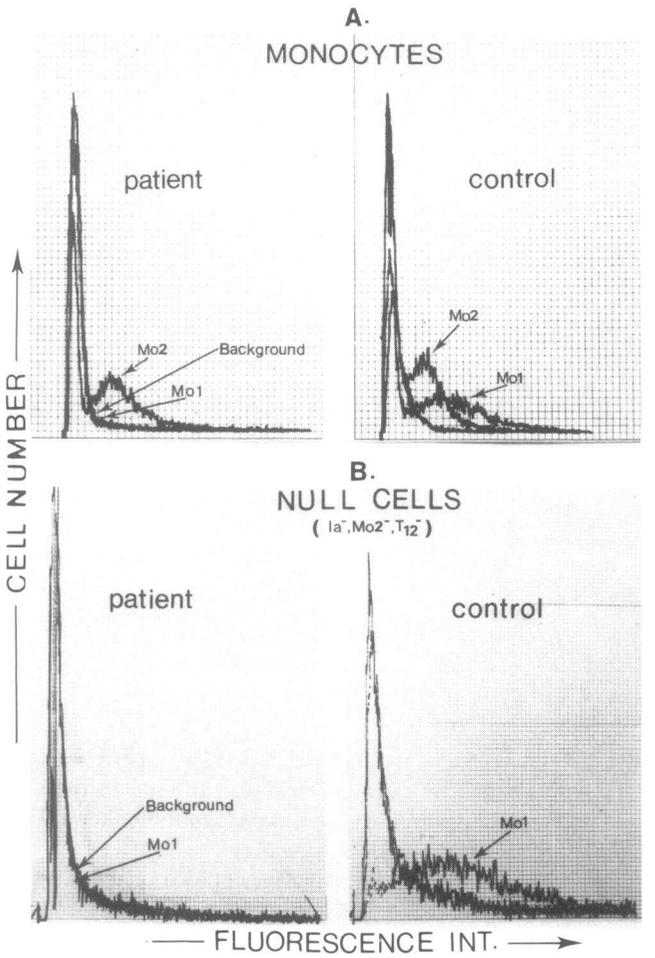

Figure 2. Immunofluorescent flow cytometry analysis of patient and control cells by using monoclonal antibodies to cell surface structures (*A*) Fluorescence staining of patient (*left*) and control (*right*) monocytes with monoclonal antibodies to Mo1 and Mo2 (monocyte specific). No fluorescence on patient's cells is noted when a monoclonal antibody to Mo1 is used. (*B*) Similar analysis reveals no fluorescence with monoclonal antibody to Mo1 on patient's null cell-enriched population.

pool and therefore, inaccessible to surface iodination or fluorescence analysis. To rule out this possibility, both normal and patient granulocytes were rendered permeable to the two subunit-specific monoclonal antibodies to Mo1 by pretreatment with methanol (15), followed by indirect immunofluorescence analysis. On normal cells, there were appreciable levels of intracytoplasmic staining in addition to that detectable on the surface membrane as seen by fluorescence microscopy and quantitated by FACS-1 cytofluorography. With the patient's cells, however, there was no measurable intracellular fluorescence over background (data not shown).

Detection of complement and F_c receptors on Mo1-deficient phagocytes. Since it was previously shown that monoclonal antibodies to Mo1 and Mac-1 specifically block C3bi receptor-mediated rosette formation with normal cells (3, 4), the ability of Mo1-deficient cells to rosette with erythrocytes coated with C3bi was examined. As can be seen in Table I, both the patient's granulocytes and monocytes rosetted normally with EIgG or EAC43b. The patient's cells also rosetted normally with EAC43bi. Percent rosetting was not inhibited by an optimal concentration of either anti-CR1 or anti-Mo1 (Table I). However, when the two antibodies were used together, no C3bi-dependent rosetting was seen with the patient's cells. Furthermore, EAC43bi binding was not mediated by an abnormally expressed receptor specific for the C3d fragment of C3 (C3d receptor) since no rosetting of EAC43d to the patient's granulocytes was seen. It appears likely, therefore, that binding of EAC43bi to the patient's phagocytes is mediated by the C3b receptor and dependent on small amounts of Mo1 antigen detected by this inhibition assay. In the presence of anti-CR1 or anti-Mo1 alone, EAC43bi binding is dependent on Mo1 or mediated by C3b receptor, respectively.

Phagocytosis and NK activity by Mo1-deficient cells. Ingestion of serum-opsonized ORO particles by the patient's monocytes was significantly depressed (Table II), a finding similar to that noted in his granulocytes (5). Ingestion of EIgG by patient's monocytes was also significantly reduced (Table II). Normal monocytes treated with anti-Mo1 showed a similar impairment in ingestion of serum-opsonized ORO or of EIgG. Inhibition of IgG phagocytosis was more striking at low IgG input (data not shown).

Since PBL enriched for null cells were deficient in Mo1 expression, it was of interest to determine if this deficiency was associated with an impairment of NK, a function attributed to M1 (Mo1) positive null cells (23). Both patient and control cells mediated comparable degrees of lysis against K562 target cells indicating that deficiency of surface Mo1 does not alter the cells ability to perform NK (Fig. 3).

Discussion

Several patients with recurrent bacterial infections associated with deficiencies of granulocyte proteins have been described (5, 24, 25, 26). We used monoclonal antibodies to further characterize the functional defects in our patient. This study demonstrates the deficiency of a surface membrane glycoprotein, Mo1, on this patient's granulocytes, monocytes, and null cells. The patient's granulocytes have an impaired capacity to ingest C3-opsonized particles such as zymosan, lipopolysaccharide-coated ORO, as well as particles coated with IgG. Zymosan-induced superoxide generation and lysozomal enzyme release are also depressed in Mo1-deficient granulocytes (5). The patient's monocytes demonstrate a similar impairment of phagocytic function (Table II). NK activity by the patient's PBL was normal (Fig. 3), in agreement with in vitro studies in which pretreating PBL with anti-Mac-1 (27) or anti-Mo1 (Todd, R. F., III, unpublished data) did not interfere with their NK activity.

Table I. Percentage of Cells Rosetting with IgG-, C3b-, or C3bi-coated Sheep Erythrocytes

			EAC43bi			
	EIgG	EAC43b	Buffer	Buffer + F(ab')$_2$ anti-CR1‡	Buffer + F(ab')$_2$ anti-Mol	Buffer + Anti-CR1 + Anti-Mol
Granulocytes						
Patient	90*	64	41	39	41	1
Control	88	65	39	44	11	2
Monocytes						
Patient	62	65	46	45	43	NT§
Control	74	72	30	30	10	NT

* Values are the means of two separate determinations. No rosetting was seen between patient or control granulocytes and unopsonized sheep erythrocytes (data not shown).
‡ Anti-CR1 was used at a concentration that produced 80% inhibition of EAC43b rosetting with granulocytes and monocytes.
§ Not tested.

On normal cells, anti-Mol, anti-Mac-1 or anti-OKM10 effectively blocked C3bi receptor-depedent rosette formation suggesting that Mol/Mac-1/OKM1 are closely associated (3, 4) or identical (28) with the C3bi receptor. It has also been suggested that EAC43bi can bind to the C3b receptor (29, 30, 31). We therefore examined the ability of the patient's phagocytes to form rosettes with EAC43bi. Percent rosetting of the patient's granulocytes and monocytes with EAC43bi in the presence of optimal concentrations of either anti-Mol or anti-CR1 alone was within normal limits (Table I). No rosetting was seen, however, in the presence of the two combined antibodies. This suggests that under the conditions used, and in the presence of anti-CR1, the small amounts of the 155-kD subunit of Mol present on the patient's cells can mediate the normal degree of EAC43bi-dependent rosetting obtained. The normal degree of rosetting seen with the patient (but not control) phagocytes in the presence of anti-Mol is not due to an increase in C3b receptor number (5) but may be due to an increased affinity of the patient's C3b receptors to C3bi. This may reflect a compensatory mechanism in Mol-deficiency states.

Structural studies revealed that the patient's cells were deficient in the two subunits of Mol antigen (Fig. 1). A monoclonal antibody to the 94-kD beta subunit of Mol/OKM1 reacts not only with Mol but also with LFA-1 (alpha subunit of 177,000-D glycoprotein) and possibly, with a third surface protein, P150,95 (alpha subunit of 150,000 D) (Fig. 1 *B*). Mol/OKM1, LFA-1, and P150,95 share the same 94-kD beta subunit (22). None of these proteins were immunoprecipitated from the patient's granulocytes by antibody to the 94-kD subunit. The patient's granulocytes and monocytes were also deficient in LFA-1 when a monoclonal antibody specific for the 177,000-D alpha subunit was used (Arnaout, M. A., C. Terhorst, J. Pitt, and

Table II. Phagocytosis by Patient and Control Monocytes

	Stimulus	
	C3-coated ORO	IgG-coated erythrocytes
	μg ORO ingested/10^7 cells/min	*% of cells ingesting*
Patient	26*	28±5‡
Control	70	64±9

* Values represent mean of two separate determinations.
‡ Values represent mean±SD of triplicate determinations.

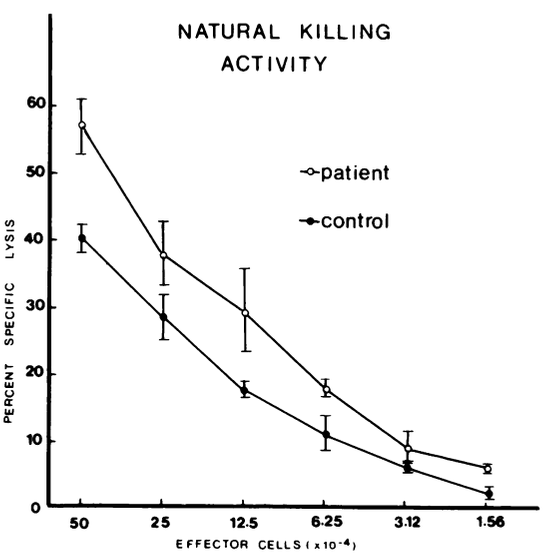

Figure 3. NK of K-562 target cells by patient and control PBL±SD (triplicate determinations). Over a wide range of effector to target cell ratios, no decrease in NK activity is noted in the Mol-deficient patient cell population. Spontaneous lysis was always <5% of total lysis. Similar results were obtained in one other experiment.

R. F. Todd III, manuscript in preparation). It is possible that deficiency of the 94-kD glycoprotein may be the primary defect in this disease. During biosynthesis, association of the 94-kD subunit with Mo1, LFA-1, and P150,95 takes place (22) and may be necessary for the stable surface expression or function of these molecules.

Monoclonal antibodies to the 155,000-D subunit of Mo1 inhibited phagocytosis of C3- or IgG-coated particles as well as opsonized zymosan-induced, lysozomal enzyme release and superoxide generation by normal cells (3) (Arnaout, M. A., and J. Melamed, unpublished observations). Inhibition of IgG-dependent phagocytosis was more pronounced at low rather than high IgG input. The role of Mo1 in IgG-dependent phagocytosis may be explained if Mo1 also functions in promoting adherence between certain surfaces and phagocytic cells (3). Of interest is that LFA-1, a structurally similar molecule, appears to serve such a function on lymphocytes (12, 32).

The anti-Mo1-induced functional defects on normal phagocytes were similar to those observed in Mo1-deficient cells. These findings suggest an association between deficiency of the 155,000-D piece of Mo1 and the observed functional defects seen in the patient's phagocytes. However, it should be noted that if deficiency of the 94-kD subunit is the primary defect, this may have a pleiotropic effect. The 94 kD is common to three different surface antigens: Mo1, LFA-1, and P150,95. Immunoprecipitation studies indicate that at least two and possibly all three are deficient from the patient's granulocytes. It is possible that deficiency of these additional glycoproteins could also contribute to the observed functional defects.

Note added in proof. Three more patients with recurrent bacterial infections and Mo1 deficiency were recently found (Ross, G. D., R. A. Thompson, M. J. Walport, R. H. Ward, J. Iida, S. L. Newman, R. A. Harrison, and P. J. Lachmann, manuscript submitted for publication).

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