The Relationship Between CR3 Deficiency and Neutrophil Actin Assembly

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Polymorphonuclear leukocytes (PMN) with a deficiency of the complement receptor type 3 (CR3) membrane glycoprotein family have impairments in the ability to adhere to surfaces as well as chemotactic and phagocytic defects, processes that require a functional contractile apparatus. PMN from the patient with neutrophil actin dysfunction (NAD) displayed similar functional characteristics to those with CR3 deficiency suggesting the two disorders may be the same disease. In order to evaluate the relationship between CR3 deficiency and actin assembly, actin filament assembly was measured in PMN from six previously reported homozygotes (two severe and four moderate CR3-deficient patients) as well as five heterozygotes for CR3 deficiency. PMN from all patients had normal unstimulated concentrations of F-actin and after exposure to the chemotactic peptide FMLP (5 x 10^{-7} mol/L for 5 to 40 seconds at 25°C) assembled actin normally. Pretreatment of normal PMN with concentrations of monoclonal anti-α CR3 antibody, capable of blocking PMN adherence, also failed to impair FMLP-induced actin filament assembly. CR3 glycoprotein expression was measured in PMNs from the mother, father, and older sister of the NAD patient (N Engl J Med 291:1093, 1974). Actin filament assembly was recently shown to be defective in PMNs from all three family members. The total concentrations of the α and β CR3 subunits were below normal in PMN detergent extracts from the mother (25% of simultaneous controls) and older sister (56% of control). PMN surface expression of these two subunits was also found to be depressed (mother, 50%; older sister, 63% of control). These findings suggest these two NAD family members are heterozygote carriers for CR3 deficiency as well as NAD. Simultaneous studies of the father, however, demonstrated normal total concentrations of both the α and β CR3 subunits (126% of controls) as well as normal surface expression of both subunits after phorbol myristate acetate stimulation and incubation at 37°C (mean, 112% of controls) but slightly lower than normal levels after FMLP stimulation (mean, 83%). These findings indicate that CR3 deficiency generally is not associated with defective actin filament assembly and support the conclusion that NAD represents a unique kindred in which PMN actin function differs from previously reported genotypes of CR3 deficiency.

A GENETIC DISORDER characterized by abnormal polymorphonuclear leukocytes (PMN) adherence, chemotaxis, and phagocytosis was described in 1980 in a young male child with severe recurrent bacterial infections. These PMN functional abnormalities were similar to those described in a patient with neutrophil actin dysfunction (NAD) described 6 years earlier. Actin filament assembly in extracts derived from diisopropylfluoro-phosphate (DFP)-treated PMNs from this new patient were found to be normal, and the biochemical basis of the disorder was attributed to the genetic deficiency in the surface expression of an adherence promoting surface glycoprotein. Subsequent work has established that this glycoprotein is representative of a family of leukocyte receptors designated CR3 (CD11b/CD18), LFA-1 (CD11a/CD18), and Gp 150,95 (CD11c/CD18) and collectively called CR3 glycoprotein family (CR3 glycoproteins) or CD11/18 glycoproteins. Each of these three antigens are heterodimers with a distinct α subunit (CD11a, b, c) noncovalently linked to an identical β subunit (CD18). Subsequently a number of investigators have identified additional cases with deficiency of this glycoprotein family. Heterogenous mutations of the common β subunit have recently been shown to result in defective surface expression of these surface heterodimers.

The association of deficient expression of the CR3 glycoprotein family and defects in phagocytosis and chemotaxis, two processes believed to require contractile protein function, raise the possibility that one or more structural abnormalities in these glycoproteins could result in defective transmission of signals regulating the actin filament cytoskeleton during agonist-induced cell movement. A relationship between the CR3 glycoproteins and PMN actin function was first suggested by the preliminary finding of reduced CR3 expression in PMNs of some family members of the NAD index case. A recent review of neutrophil disorders has suggested that NAD and CR3 deficiency are the same disease; however, with the exception of the first reported CR3-deficient patient, PMN actin function has not been examined in patients with this glycoprotein deficiency. Therefore, PMN actin filament assembly was tested in 11 previously reported individuals with partial or complete CR3 deficiency and CR3 expression carefully assessed in the remaining relatives of the NAD index case.

METHODS

Patients. Homozygotes for CR3 deficiency included two patients with a severe phenotype as classified by Anderson et al.

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Submitted October 3, 1988; accepted January 20, 1989.

Supported in part by Grants No. RO1 AI 23262-02 and AI 21963 from the National Institute of Health, Bethesda, MD, and March of Dimes Grant No. 1010.

M.A.A. was an Established Investigator of the American Heart Association.

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0006-4971/89/7307-0023$3.00/0

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Patient no. 1 in our study, an 8-year-old girl (patient no. 1, reference 12) and patient no. 2, a 5-year-old boy (patient no. 9, reference 4) and four patients with a moderate phenotype: patient no. 3, a 9-year-old boy (patient no. 7, reference 12); patient no. 4, a 20-year-old man (patient no. 4, reference 12); patient no. 5, a 9-year-old boy reported by Arnaout et al; and patient no. 6, a 38-year-old man (patient no. 6, reference 12). Two heterozygotes for CR3 deficiency from the above kindreds (the mother of patient no. 1 and the sister of patient no. 4) were studied. In addition, three heterozygotes (the mother, father, and sister) from another kindred were investigated. Although preliminary evidence suggested that the homozygote from this family (infant no. 2, reference 19) had both a neutrophil actin defect and a near-complete deficiency of CR3, subsequent analysis indicated no definitive evidence for defective PMN actin filament assembly. He has, therefore, been classified as a severe phenotype for CR3 deficiency. PMNs from these five relatives had approximately 50% of the normal CR3 α and β subunits as assessed by fluorescent antibody staining (see below). All of the heterozygotes were clinically well and had no history of recurrent infections. Family members of the index case with NAD have been described. They also have had no history of recurrent infections. FMLP stimulated PMN from the father, mother, and older sister (sister I) have significantly lower actin filament content than controls as measured by the NBD-phallacidin staining and Triton-insoluble cytoskeleton assays (Figs 1 through 3).

**Immunoblots.** Western blots were performed as described by Towbin et al. PMNs, 2.5 × 10⁹, were treated with 5 mmol/L DFP, followed by suspension in phosphate buffered saline (PBS) containing 0.5% NP40 and 2 mmol/L phenylmethylsulfonyl fluoride (PMSF). Samples were then incubated on ice for 15 minutes.
followed by centrifugation at 100,000 g in a Beckman airfuge for 30 minutes. The resulting supernatants were electrophoresed in 1% sodium dodecyl sulfate (SDS) through 7.5% polyacrylamide slab gels and then transferred to nitrocellulose membranes by electrophoresis. The filter was then blocked with PBS containing 20% nonfat milk for two hours at room temperature and then incubated with rabbit anti-human Mol α (CD 11b) or Mol β (CD 18) antibody (1:500 dilution) or a nonreactive rabbit IgG on a mixing platform overnight at 4°C. The filter was then washed and incubated with 125I-labeled goat anti-rabbit immunoglobulin (New England Nuclear, Boston) for one hour at room temperature. The washed filter was then dried and exposed to XOR matic film overnight at 70°C with an enhancing screen.

Immunofluorescence studies. Determination of surface α and β subunits concentrations of CR3 and CR1 using FACS were performed as previously described. PMNs were incubated with final concentrations of 5 x 10⁻⁸ and 5 x 10⁻⁹ mol/L FMLP, 1 x 10⁻⁹ mol/L PMA or buffer alone at 37°C for 30 minutes and 15 minutes, respectively, before immunofluorescent antibody staining. Antibodies used for these assays included monoclonal antibodies OKM1 anti-Mac-1 α (CD 11b)(9), Tsl/18 anti-β(CD 18)(9), 44 Mol anti-α(CD 11b)(3), L11 anti-LFA 1 α (CD 11a), Leu M 5 anti-Gp 150 (CD 11c), and 3D9 anti-CR1.

Other procedures. Isolation of human granulocytes in intact PMNs for the nitrobenzoazidolazol (NBD)-phallacidin assay were prepared by hypotonic lysis of whole blood as previously described. Cells were fixed, permeabilized, and stained using the method of Howard and Oresajo.

Fluorescent values in all actin experiments were within the linear range for both machines, therefore, values could be normalized so that the mean unstimulated control value was 32 fluorescent channel. Simultaneous stimulated control, as well as unstimulated and stimulated patient values were then multiplied by the same correction factor. Determinations of the concentrations of actin associated with the Triton X-100 insoluble PMN cytoskeletons were performed using a modification of the method of White et al as recently described.

The NBD-phallacidin and Triton-insoluble cytoskeleton assays were used rather than the previously reported sedimentation assay because these two newer assays measure actin function in intact cells rather than in homogenized extract. They also measure PMN actin polymerization in response to a physiological stimulus (FMLP) as compared with the sedimentation assay, which requires incubation in an unphysiologic salt concentration (0.6 mol/L KCl) to induce filament assembly. Finally, unlike the other two assays, the sedimentation assay is significantly affected by serine protease activity.

FMLP was chosen as the agonist for these experiments rather than opsonized zymosan (C3bi or CR3 receptor mediated) because FMLP receptors have been demonstrated to be normal in CR3-deficient patients and in NAD family members. A defect in PMN actin assembly following FMLP stimulation would therefore reflect an abnormality in signal transduction or actin function rather than a receptor deficiency.
PMN Actin filament formation in CR3-deficient patients. To investigate a possible association between deficiency of the CR3 surface glycoproteins and defective cytoplasmic actin function, PMN actin filament formation was studied in six homozygotes and five heterozygotes with CR3 deficiency. These patients have all been previously described.4,5,2,9 Figure 1 shows PMN actin filament (F-actin) content of a representative homozygote, heterozygote, control and mean values for three NAD family members as measured by NBD-phallacidin staining. Unlike the recently described NAD family members,19,22 whose PMN had low resting F-actin content (mean fluorescence 23.6 ± 0.4 SEM, three patients), unstimulated PMN F-actin content in homozygotes (30.9 ± 2.2, five patients), and heterozygotes (33.7 ± 2.4, five patients) was found to be comparable with controls (32 ± 3.5, N = 10) (Fig 2A). The rise in F-actin induced by FMLP (1 x 10^-7 mol/L, final concentration) stimulation was comparable with controls (30 seconds, 72.2 ± 2.5, ten controls) in both homozygotes (75.3 ± 0.8, five patients) and heterozygotes (81.3 ± 4.7, five patients) (Fig 2B). These levels were significantly higher than NAD family members (52.7 ± 1.3, three patients). The rates of actin polymerization following FMLP stimulation were somewhat higher in the two patients with severe CR3 deficiency (7.1% and 8.0%/s) than controls (5.7% ± 0.3%/s, N = 9, range 4.4% to 6.9%/s). Rates of the three moderate homozygotes and five heterozygotes (5.1% to 6.8%/s) were all within the normal range as were NAD family members (4.5% ± 0.3%/s, three patients) (Fig 2C).

Concentrations of actin associated with the Triton-insoluble cytoskeleton were determined for four homozygotes and two heterozygotes. As observed with the NBD-phallacidin assay, PMN actin filament content after 10 and 40 seconds stimulation with FMLP was comparable with controls (10 seconds; mean, 1.62 ± .04 SEM; range, 1.47 to 2.10, N = 14; 40 seconds; mean, 1.69 ± .07; range, 1.43 to 2.3, N = 12) (Fig 3). All patient values were within the normal range with the exception of the 10-second time point (1.40) of patient no. 5, a moderate homozygote; however, at 40 seconds his PMN had higher concentrations of Triton-cytoskeleton associated actin (2.1) than all but one control. All CR3-deficient patients studied had significantly higher F-actin content at both time points than NAD family members (Fig 3).

In addition to actin, the relative concentrations of nine other oligopeptide bands visualized on one-dimensional SDS polyacrylamide gel electrophoresis (PAGE) were quantitated in control Triton-insoluble PMN extracts and CR3 deficient patient PMN extracts. No significant differences between patients and controls were found.

CR3 and CR1 receptor studies of NAD granulocytes. To determine if the heterozygotes for NAD, shown to have a partial defect in PMN actin filament assembly, might also have a deficiency of CR3, nitrocellulose immunobLOTS of whole cell PMN extracts from the parents and sister of the NAD index case were performed. Studies using anti-CR3 antibodies directed against the α or β subunit, demonstrated partial deficiencies of both subunits in the mother and older sister. By this assay the father and younger sister (sister 2) had normal concentrations the CR3 α antigen. The father also demonstrated normal concentrations of the β subunit while sister 2 had 60% of normal concentrations. The molecular weights of the two subunits as determined by migration on SDS-PAGE were comparable with controls (Fig 4, Table 1). These findings were confirmed by immunofluorescence flow cytometry. Using fluorescently labeled OKM1 anti-Mac-1 α, anti-Mol α (CD11b), and TS1/18 anti-β (CD18) monoclonal antibodies a partial deficiency of both surface glycoprotein subunits was again seen in the mother and sister 1, suggesting they were heterozygotes (Fig 5, Table 1). Mild decreases in the surface expression CD11a (79% of control in the mother and 74% of control in sister 1) and CD11c (95% in mother and 78% of control in sister 1) were also observed in PMN stimulated with FMLP (1 x 10^-7 mol/L for 20 minutes) and were comparable with previously studied heterozygotes for CD11/18 deficiency.12,14 The father had near normal concentrations of both CD11b and CD18. Following stimulation with FMLP (1 x 10^-8 to 1 x 10^-7 mol/L) the father’s values were slightly lower than normal, the CD11b averaging 80% of simultaneous controls (range, 79% to 80%) and CD18 averaging 86% of controls (range, 82% to 90%, three determinations). Following warming to 37°C or exposure to PMA, surface concentrations of the CD11b and CD18 subunits were found to be normal (mean, 114%; range, 105% to 127% of control, four determinations). After incubation with FMLP, expression of CD11a and CD11c were also within normal limits (respectively, 128% and 133% of controls). Sister 2’s values for CD11b and CD18 were consistently lower than normal (mean, 76%; range, 62% to 85% of control, five determinations), although generally not as low as sister 1 (mean, 63%; range, 61% to 66% of controls).

CR1 surface antigen concentration was also measured by immunofluorescence flow cytometry using 3D9, a monoclonal antibody directed against complement receptor type I (CR1). PMN membrane concentrations in the mother, father, and sister 1 were found to be comparable with controls.

Effects of anti-CR3 antibody on actin filament assembly in normal PMNs. Normal PMNs were incubated for ten minutes at 37°C with Fab fragments derived from monoclonal anti-Mol α IgG (CD11b). The final concentration of antibody used (40 μg/mL) previously had been shown to block completely homotypic PMN adherence in response to FMLP.30 Pretreatment with this antibody failed to impair FMLP-induced PMN actin polymerization as measured by the Triton-insoluble cytoskeleton assay (control PMNs, F-actin content after 40 seconds stimulation with 1 x 10^-7 mol/L FMLP: 1.49, Mol treated PMNs: 1.63).

Discussion

The clinical and PMN functional similarities between deficiency of the CR3 glycoprotein family and neutrophil actin dysfunction raise the possibility that these two disorders might also have a similar biochemical basis and that the CR3 deficiency might be generally associated with defective PMN actin assembly. Investigations of seven CR3-deficient kindreds demonstrated normal PMN actin assembly as...
Fig 4. Autoradiograms of immunoblots of the NP-40 soluble fractions derived from PMNs of NAD family members and a control. PMNs, 2.5 x 10^6, were solubilized with NP40 followed by centrifugation at 100,000 g as described in the text. Supernatants were then electrophoresed in SDS through polyacrylamide slab gels, transferred to nitrocellulose, which was reacted (a) with anti-Mo1 polyclonal anti-alpha antibody and (b) anti-beta antibody as described by Towbin et al. PMN lysates from the father (F) of the index NAD patient had normal amounts of Mo1alpha and beta subunits that were comparable to the control (C). Both the mother (M) and older sister (S1) had reduced levels of both subunits. The younger sister (S2) had normal amounts of the alpha subunit but slightly reduced levels of the beta subunit. (Densities were quantitated by laser densitometry, see Table 1.) The second 60,000 molecular weight band (arrowhead) identified by anti-a antibody has not been identified. Note equivalent concentrations of this protein were found in all PMN extracts suggesting comparable loads of total protein.

Table 1. Quantitation of Surface and Total CR3 alpha and beta Subunit Concentrations by Fluorescence-Activated Cell Sorter (FACS) and Immunoblots

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<td>Father</td>
<td>80 (3)</td>
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<td>Sister 2</td>
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PMNs were incubated in buffer containing 1 x 10^-8 or 1 x 10^-9 mol/L FMLP, PMA 1 x 10^-9 mol/L, or in buffer alone for 30 minutes at 37°C. Antibodies used were monoclonal OKM1 anti-Mac-1 alpha, TS1/18 anti-beta, Mo1 anti-alpha, and 3D9 anti-CR1. Numbers in parentheses represent the number of determinations.

*Data expressed as percentage of averaged controls run in parallel with the patients. Mean fluorescence intensity was measured for FACS; relative density of the polypeptide bands on autoradiograms shown in Fig 5 were determined by a soft laser densitometer and the areas under absorbance peaks integrated.

measured by our assays, indicating that in the majority of cases CR3 deficiency is not associated with a neutrophil actin dysfunction. In addition, lack of inhibition of actin function by pretreatment of normal PMNs with anti-Mo1 alpha (CD11b) antibody indicate that inhibition of PMN adhesion is not necessarily associated with impaired actin filament assembly. It remains possible that these CR3-deficient kinreds have a more subtle abnormality in their contractile apparatus or in actin filament assembly that was not detected by the assays used. However, if such a defect is present, it is probably distinct from that found in neutrophil actin dysfunction since these same assays did demonstrate defective actin function in the NAD kindred.

The unexpected coexistence of CR3 deficiency and actin dysfunction in members of the NAD family indicate that in certain forms of CR3 deficiency actin function may be impaired. Since at least six genetic defects have been shown to result in deficiency of these surface glycoproteins, it is possible that a unique structural abnormality in one of these glycoproteins could have resulted in defective membrane receptor coupling to the actin filament cytoskeleton in these NAD relatives. The normal mobility on SDS-PAGE of the CR3 alpha and beta subunits in these individuals makes a gross structural defect unlikely but does not rule out a small deletion or point mutation. The dissociation between normal to near-normal CR3 levels in the father’s cells and his heterozygote status with respect to NAD might be explained by a defective beta subunit that is normally expressed on the
that derangements in actin assembly could lead to reduced CR3 expression in NAD. This hypothesis seems unlikely, since both the father and mother had comparable defects in actin filament assembly but markedly different PMN concentrations of CR3 antigen. A more generalized depression of receptor expression might be expected if the cytoskeletal actin played an integral role in surface glycoprotein processing and expression; however, CR1 levels were found to be normal in the father, mother, and sister 1 of our index case, and FMLP receptor function in both the mother and father was shown to be normal as assessed by degranulation.

The slightly lower levels of surface CD11b and CD18 noted in the PMN from the father following FMLP stimulation may suggest a mild defect in upregulation of this glycoprotein receptor in response to this stimulus, but not to PMA or warming. Our investigations of surface receptor concentration and function in NAD heterozygotes indicate that at least a partial defect in actin filament assembly does not significantly impair receptor expression. It remains possible that a near-complete defect in PMN actin function as described in the original NAD index case could lead to a severe deficiency of these membrane glycoproteins.

A third explanation for our observations is that CR3 deficiency and NAD in this family have both been caused by a single DNA segment deletion. Such a deletion would have to include regulatory elements for synthesis of both CR3 and an actin-modulating protein important for PMN actin polymerization.

The association of CR3 deficiency with neutrophil actin dysfunction raises the possibility of, but as outlined above does not prove, a functional link between the CR3 receptor and actin filament assembly. These investigations do indicate that in the majority of CR3-deficient patients PMN actin filament assembly as measured by our assays is normal and suggests that NAD represents a unique CR3-deficient kindred.

ACKNOWLEDGMENT

We thank Dr T.A. Springer and Melanie Miller for performing FACS analysis on the NAD family members, Dr D. Wang and G. Dabiri for assisting in PMN actin polymerization studies, and Dr F. Schmalstieg for providing CR3-deficient patients.

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