THE SEQUENTIAL APPEARANCE OF Ia-LIKE ANTIGENS AND TWO DIFFERENT COMPLEMENT RECEPTORS DURING THE MATURATION OF HUMAN NEUTROPHILS*

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The differentiation of neutrophils in normal, abnormal, and leukemic disease states has been extensively characterized from the aspects of morphology, cytochemistry, and functional properties. However, in comparison to the many studies of membrane components as indices of differentiation in the lymphoid system, little attention has been given to analysis of the membrane structural changes that occur during granulocyte differentiation. Since the acquisition of receptors and other mature cell membrane components is closely associated with functional differentiation, it is likely that identification of the structures and receptors associated with membrane differentiation would provide additional perspectives of particular utility in studies of granulocyte physiology, as well as assistance in classification of certain abnormal disease states.

Mature neutrophils have complement (C) receptors that are believed to be exclusively CR1 (immune adherence, C4b-C3b receptors) but not CR2 (C3d receptors) (1-4). However, there have been no studies of the specificity of neutrophil C receptors for different fragments of C3 or C4 molecules, or of when C receptors appear during neutrophil maturation. By contrast, the specificity

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Abbreviations used in this paper: AML, acute myelogenous leukemia; B lymphocyte or B cell, bone marrow-derived lymphocyte; T lymphocyte or T cell, thymus-derived lymphocyte; BSA, bovine serum albumin; band or band form, neutrophil maturation stage proceeding the polymorphonuclear stage; C, complement; CR1, complement receptor type one, the immune adherence (C4b-C3b) receptor; CR2, complement receptor type two, C3d receptor; C4b, the 204,000 dalton fragment of C4 generated by cleavage of C4 by C1; C3b, the 181,000-dalton fragment of C3 generated by cleavage of C3 by C42 or trypsin; C1c (140,000 daltons) and C3d (30,000 daltons) fragments are the fragments of C3b generated by cleavage with C3b inactivator, trypsin, or elastase; CML, chronic myelogenous leukemia; EAC, sheep erythrocyte-rabbit IgM antibody-complement complexes; Fc receptor, receptor for the Fc portion of IgG; HBSS, Hanks' balanced salt solution; Ia antigens, human analogues of murine "immunity associated" antigens; polymorphonuclear neutrophil, neutrophil with segmented nucleus; PBS, phosphate-buffered saline; PBS/BSA, PBS containing 1% BSA and 0.2% NaN3; SDS, sodium dodecyl sulfate.
and activity of IgG Fc receptors have been studied in greater detail, and parallel studies of murine neutrophil maturation have now demonstrated that Fc receptors appear at an early phase of neutrophil differentiation that precedes the appearance of C receptors (5).

In a companion study, immature normal human granulocyte precursors, including cells responsible for granulocyte-monocyte colony-forming cells, were recently demonstrated to express Ia determinants in a membrane surface 29,000–37,000 dalton bimolecular complex identical to that found on B lymphocytes (6). In the present investigation, the specificity and characteristics of the different types of neutrophil C receptors are examined. Emphasis is placed on determining the interrelationships of C-receptor appearance with the expression of Ia antigens during the process of normal and leukemic granulocyte maturation.

Materials and Methods

Human Subjects. Blood was obtained from normal healthy volunteers and patients with myeloid leukemia. Bone marrow was obtained from ribs removed during thoracotomy from nonleukemic individuals. Patients with myeloid leukemia were classified by standard morphological and cytochemical criteria. All patients with chronic myelogenous leukemia (CML) were shown to have the Philadelphia chromosome.

Separation of Cells by Discontinuous Density Gradient Centrifugation. Heparinized blood was mixed with an equal volume of 3% dextran T-500 (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) in phosphate-buffered saline (PBS) and after sedimentation of erythrocytes for 30 min at room temperature, the leukocyte-rich supernate was diluted with an equal volume of PBS. Marrow was flushed from 2.5-cm rib segments with Ca++ and Mg++-free Hanks' balanced salt solution (HBSS) containing 3.8% sodium citrate, 3.5% bovine serum albumin (BSA) and 20 U/ml deoxyribonuclease (Sigma Chemical Co., St. Louis, Mo.). A stock solution of 20% Hypaque-M (Winthrop Laboratories, New York) and 15% Ficoll 400 (Pharmacia Fine Chemicals) in water (d = 1.15–1.16 g/ml, 20°C) was prepared and diluted with water to make various densities for cell separation. Leukocyte suspensions were distributed into four groups of 24 × 105-mm siliconized glass centrifuge tubes, 6 ml per tube. The first group of tubes was underlayered in succession with 6-ml layers of 1.05, 1.06, and 1.07 g/ml Ficoll-Hypaque. The Ficoll-Hypaque was pumped beneath the cell suspension with a syringe pump (model 351, Sage Instruments Div., Orion Research Inc., Cambridge, Mass.) connected to capillary tubing that extended to the bottom of each tube. The second group of tubes received 1.07–, 1.08–, and 1.09-g/ml layers, and the third group 1.09, 1.105 and 1.12 g/ml Ficoll-Hypaque. The fourth group of tubes received a single layer of 1.12 g/ml Ficoll-Hypaque. After centrifugation at 1,200 g for 45 min at 20°C, the cell layers concentrated at each density interface were aspirated with a siliconized Pasteur pipet. The top layers of tube groups two and three were heterogeneous with respect to cell density and were discarded. The 1.12-g/ml layer from the fourth group of tubes contained >98% of the nucleated cells and few, if any, erythrocytes. This cell layer was used as the source of unseparated cells. After one wash of each density layer with PBS containing 1% BSA and 0.2% NaN3 (PBS/BSA), cell counts were made to determine yield, and cytocentrifuge preparations were stained with Wright-Giemsa to determine differential morphology.

C-Receptor Assay. EAC (sheep erythrocytes, sensitized with rabbit IgM antibody and complement) were prepared as previously described with the purified components of human C (7). The EAC1b used to form CR1-dependent rosettes were prepared with 50 µg of purified C4 (8) per 10⁹ EAC1, and produced nearly equivalent immune adherence as did the EAC1-3b. The EAC1-3d had <2% immune adherence activity compared to EAC1-3b, and were frequently completely negative in immune adherence. The potency of the EAC1-3d for detection of CR1 was assessed by rosette formation with cells from a lymphoblastoid cell line that contained only CR1 and lacked CR1 (Daudi).

C-receptor-dependent rosettes were formed in 10 × 75-mm plastic tubes by mixing together 0.1 ml of EAC at 2 × 10⁹/ml with 0.1 ml of C-receptor-bearing cells at 4 × 10⁹/ml in PBS/BSA,
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followed by incubation on a tube rotator for 15 min at 37°C (7). Rosettes in the cell suspension were enumerated by phase-contrast microscopy, or alternatively, differential counts of rosetted cells were made from Wright-Giemsa-stained smears. For staining, rosette mixtures were pelleted at 100 g for 2 min, resuspended in 20 µl of fetal calf serum, and immediately smeared across microscopy slides with a cover slip. The smears were quickly dried with a hair drier and then stained with Wright-Giemsa. By this technique, an even distribution of cells with good morphology was obtained, and rosettes of cells representing different stages of myeloid cell maturation could be easily distinguished (6).

Detection of Human Ia Antigens. Human Ia antigens were detected by direct immunofluorescence with a rabbit F(ab')2 antibody conjugated to tetramethylrhodamine isothiocyanate as previously described (6, 9).

Preparation of C Fragments. Highly purified C4 and C3 were prepared by the recently published techniques of Bolotin et al. (8) and Tack and Prahl (10). C4b was separated from native C4 on DEAE-Bio-Gel A (Bio-Rad Laboratories, Richmond, Calif.) during the final step of the C4 purification procedure (8). In agreement with the findings of Cooper (11) and Bolotin et al. (8), the α-chain of reduced C4b was shown by electrophoresis in sodium dodecyl sulfate (SDS)-polyacrylamide gels to be 6,000 daltons less molecular weight than the α-chain of native C4, whereas the β- and γ-chains of C4b had molecular weights identical to those of native C4. C3b was prepared according to Bokisch et al. (12) by cleavage of C3 with 1% trypsin for 2 min at 20°C. C3b was separated from C3a by gel filtration on Sephacryl S-200 (Pharmacia Fine Chemicals) in 0.05 M sodium acetate, pH 5.8, containing 1.0 M sodium chloride (B. F., Tack, personal communication). This latter step did not cause the denaturation and precipitation of C3b that characterized the original separation technique (12) using 0.15 M NaCl at pH 4.0. C3b was shown to be free of C3a, C3c, and C3d by electrophoresis in SDS-polyacrylamide gels. Ouchterlony double-diffusion analysis with a rabbit anti-C3d serum gave a line of identity between C3b and C3d, indicating that C3d determinants were exposed in the C3d region of fluid-phase C3b. This activity of the anti-C3d against C3b was unaffected by absorption of the antisemum with C3c immobilized with cyanogen bromide on Bio-Gel A-5m (Bio-Rad Laboratories). Furthermore, the anti-C3d had a similar activity with immune complex-bound C3b and C3d as it did with the fluid-phase fragments. Anti-C3d agglutinated both EAC1-3d and EAC1-3b to a titer of 1:2,400. Thus, this antisemur demonstrated that the C3d region was exposed in both fluid-phase and immune complex-bound C3b. C3c was a generous gift from Dr. Brian Tack of the National Institutes of Health, Bethesda, Md., and was produced by cleavage of C3 with 1% porcine elastase for 4 h at 37°C, followed by separation from C3d on Sephadex G-100 in 0.05 M sodium acetate pH 5.6 containing 1.0 M NaCl. The C3c produced by this method was free of detectable C3b and C3d by immunodiffusion and SDS-polyacrylamide gel electrophoresis analysis. In immunodiffusion tests, elastase-generated C3c formed a line of identity with trypsin-generated C3c and anti-C3, but did not form a precipitin line with anti-C3d produced by immunization with trypsin-generated C3d. Although the molecular weights of the unreduced C3c fragments were similar, elastase-generated C3c differed from trypsin-generated C3c with respect to the specific cleavage sites in the C3b α'-chain. Most importantly, few other peptides in the α'-chain were cleaved with elastase, and unlike trypsin, elastase did not cleave the β-chain. C3d was produced by cleavage of C3 with 2% trypsin at 37°C for 1 h, followed by separation from C3c and C3b by filtration on Sephadex G-200 in Veronal-buffered saline (12). No C3a, C3b, or C3c were detected in the C3d by SDS-polyacrylamide gel electrophoresis or by immunodiffusion.

Assay for C-Receptor Specificity. The C receptors of neutrophils were analyzed for specificity by tests for competitive inhibition of EAC rosette formation with fluid-phase fragments of C4 and C3. 4 x 10⁶ neutrophils were resuspended in 0.1 ml of C-fragment dilution in PBS/BSA and incubated at 37°C for 15 min. Next, 0.1 ml of EAC was added to the neutrophil suspension and the mixture was analyzed for inhibition rosette formation by comparison to a simultaneous control in which PBS/BSA was substituted for the C-fragment dilution.

Results

Separation of Myeloid Cells of Different Maturation Stage by Density Gradient Centrifugation. Ficoll-Hypaque gradients containing seven density steps were used to separate myeloid cells according to the maturation stage associated
with different densities. The majority of neutrophils in normal blood were characterized by a buoyant density between 1.09 and 1.12 g/ml (Table I). The pellet fraction (>1.12) contained <10% of the total neutrophils, and the majority of eosinophils and erythrocytes. The neutrophil recovery from the gradients was 75–85% and the purity of neutrophilic cells in the 1.105- and 1.12-g/ml fractions was 95–99%, with 1–5% contamination with lymphocytes and eosinophils. Cell fractions obtained at a density of 1.07–1.08 g/ml contained 5–10% metamyelocytes and band forms, 1–3% basophils, the majority of lymphocytes and monocytes, but few polymorphonuclear cells. These fractions resembled the mononuclear cell fractions obtained by conventional Ficoll-Hypaque centrifugation according to Beyum (13).

Myeloid cells from normal bone marrow sedimented according to the degree of maturation, so that myeloblasts and promyelocytes were concentrated in low density fractions, whereas polymorphonuclear neutrophils were restricted to high density fractions (Table I). Cell density increased with maturation such that myeloblasts were isolated at 1.06-1.07 g/ml, metamyelocytes at 1.07-1.08 g/ml, band forms at 1.08-1.105 g/ml, and polymorphonuclear cells at 1.09-1.12 g/ml. The vast majority of lymphocytes and nucleated erythroid cells in the 1.05-1.06 g/ml fractions were small mononuclear cells that were easily distinguished from larger myeloblasts and promyelocytes in phase-contrast microscopy.

Blood from patients with stable-phase CML contained 0.5-3.0 × 10⁶ neutrophils.
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**Table II**

Separation of Blood Leukocytes from Patients with Myelogenous Leukemia by Density Gradient Centrifugation

| Blood source | Density layer | Proportion of total cells | Wright-Giemsa morphology* | % | % |
|--------------|---------------|----------------------------|---------------------------|---|---|
| Patient with CML | 1.05 | 0.1 | 22 | 41 | 15 | 3 | 2 | 1 | 2 | 13 |
| 1.06 | 5.2 | 4 | 21 | 39 | 13 | 8 | 2 | 6 | 7 |
| 1.07 | 25.0 | 0 | 2 | 30 | 58 | 8 | 1 | 1 | 0 |
| 1.08 | 11.1 | 0 | 0 | 14 | 59 | 18 | 5 | 1 | 3 |
| 1.09 | 18.3 | 0 | 0 | 10 | 33 | 54 | 0 | 0 | 0 |
| 1.105 | 45.3 | 0 | 0 | 7 | 12 | 78 | 1 | 0 |
| 1.12 | 1.8 | 0 | 0 | 2 | 10 | 88 | 0 | 0 |
| Unseparated | 100 | 0 | 0 | 3 | 15 | 69 | 0 | 0 |
| Patient with AML | 1.05 | 11.4 | 84 | 0 | 0 | 2 | 1 | 0 | 0 | 8 |
| 1.06 | 10.5 | 88 | 14 | 6 | 2 | 4 | 2 | 2 | 3 |
| 1.07 | 81.4 | 47 | 12 | 5 | 10 | 7 | 5 | 4 | 10 |
| >1.07 | 10.7 | 32 | 12 | 15 | 10 | 4 | 10 | 2 |
| Unseparated | 100 | 49 | 10 | 10 | 8 | 6 | 5 | 6 | 5 |

Abbreviations are explained in the legend to Table I.

*300 cells counted in each density layer; thus 0% implies <0.3%.
†No cells in layers 1.08, 1.09, 1.105, or 1.12 g/ml. The >1.07 fraction was the cell pellet in a tube containing 1.05-, 1.06-, and 1.07-g/ml layers. See abbreviations in Table I.

phils/ml, the majority of which were polymorphonuclear (Table II). Because only 0.1–1.5% of blood cells from these patients were myeloblasts and promyelocytes, density separation proved to be of considerable value in obtaining sufficient numbers of immature cells for surface marker analysis. Low density fractions contained 4–77% myeloblasts and promyelocytes, an enrichment of 50- to 300-fold compared with the unseparated leukocytes.

Blood from patients with acute myelogenous leukemia (AML) contained 50–80% myeloblasts and promyelocytes. Over 50% of these cells had a density <1.07 g/ml (Table II), however as many as 10% of the myeloblasts from AML patients often exhibited a more heterogeneous density distribution than those isolated from either normal bone marrow or patients with CML (regardless of whether they were in stable phase or blast phase).

**EAC Rosette Formation with Normal Neutrophils.** An average of 17% of normal blood neutrophils formed rosettes with EAC1-3d. This proportion of neutrophil rosettes with these immune adherence (human E rosette)-negative EAC1-3d was unaffected by retreatment of the EAC1-3d with C3b inactivator (and β1H) two times. However, when normal blood neutrophils were separated by density gradient centrifugation, the small proportion of neutrophils forming EAC1-3d rosettes was concentrated to 51% in the 1.09-g/ml fraction, and the highest density 1.12-g/ml fraction formed only 6% EAC1-3d rosettes (Table I). Thus, the neutrophils forming EAC1-3d rosettes were of lower density than the majority of neutrophils, and the proportion of neutrophils forming EAC1-3d rosettes decreased with increasing cell density. The majority of neutrophils had a density ≥ 1.105 g/ml and did not form EAC1-3d rosettes. By contrast, >90% of normal blood neutrophils formed rosettes with either EAC14b or EAC1-3b, and EAC14b and EAC1-3b rosette formation increased slightly with increasing cell density, from 82% at 1.09 g/ml to 95% at 1.12 g/ml.
TABLE III

Competitive Inhibition of C Receptors on High-Density Polymorphonuclear Neutrophils
by Fluid-Phase Complement Fragments

| Source and description of neutrophils | EAC rosette type | Concentration of C fragment μg/ml | Inhibition of rosette formation by fluid phase |
|--------------------------------------|-----------------|----------------------------------|-----------------------------------------------|
| Normal blood                         | EAC14b          | 400                              | 74 98 57 ND*                                  |
| 1.12 g/ml density layer              |                 | 200                              | 60 72 35 -4                                  |
| 98% polymorphonuclear                |                 | 100                              | 31 46 22 -2                                  |
| Rosette formation                    |                 | 50                               | 12 20 8 1                                    |
| EAC14b = 95%                         | EAC1-3b         | 400                              | 61 71 56 ND                                  |
| EAC1-3b = 97%                        |                 | 200                              | 35 53 29 -4                                  |
| EAC1-3d = 2%                         |                 | 100                              | 24 32 16 0                                   |
| EAC1-3d                              |                 | 50                               | 7 12 4 3                                     |

* ND, not done.

Among normal marrow myeloid cells, 25% formed rosettes with EAC1-3d and 44% formed rosettes with either EAC14b or EAC1-3b. Compared with normal blood, the 1.09- to 1.12-g/ml density fractions of normal marrow were heterogeneous in neutrophil morphology, containing lower proportions of polymorphonuclear cells and higher proportions of immature neutrophils. The marrow 1.12-g/ml fraction resembled the 1.12-g/ml fraction of normal blood in that the proportion of EAC1-3d rosettes was considerably less than in the 1.09- and 1.105-g/ml fractions, and the majority of cells in the 1.12-g/ml fraction formed rosettes only with EAC14b and EAC1-3b (Table I). However, the marrow 1.12-g/ml fraction represented only 5% of marrow cells, as compared to 20% of normal blood leukocytes. The majority of marrow myeloid cells were lower density and less mature than blood neutrophils and did not form EAC rosettes.

Specificity and Number of Different Types of Neutrophil C Receptors. Two different neutrophil fractions were examined for competitive inhibition of EAC rosette formation by fluid-phase fragments of C4 and C3. One fraction consisted of normal blood polymorphonuclear neutrophils isolated at 1.12 g/ml, that formed a high proportion of rosettes with both EAC14b and EAC1-3b, but very few rosettes with EAC1-3d (Table III). The other neutrophil fraction was a 1.08-g/ml density fraction, that formed similar proportions of rosettes with all three EAC types. This latter fraction was obtained from the blood of a patient with CML and consisted of 60% polymorphonuclear neutrophils, 30% band forms, and 10% immature myeloid cells (Table IV). This cell preparation was chosen because the large number of cells available permitted the assay of many different C-fragment dilutions with each EAC type. Similar results were also obtained on a smaller scale when normal marrow cells of similar density and composition were used instead of leukemic cells.
TABLE IV

Competitive Inhibition of C Receptors on Low-Density Polymorphonuclear and Band Neutrophils by Fluid-Phase C Fragments

| Source and description of neutrophils | EAC rosette type | Concentration of C fragment | Inhibition of rosette formation by fluid phase |
|--------------------------------------|------------------|----------------------------|-----------------------------------------------|
|                                      |                 | µg/ml                      | C4b  | C3b  | C3c  | C3d  |
| Leukemic blood                       | EAC14b          | 400                        | 65   | 78   | 26   | -6   |
| 1.08 g/ml density layer              |                 | 200                        | 44   | 64   | 13   | -7   |
| 30% bands, 60% polymorphs            |                 | 100                        | 35   | 55   | 6    | -3   |
| Rosette formation                    | EAC14b = 62%    | 50                         | 28   | 51   | 8    | -2   |
| EAC1-3b = 73%                        |                 |                            |      |      |      |      |
| EAC1-3d = 55%                        |                 |                            |      |      |      |      |

| EAC1-3b                              | 400              | 20                         | 61   | 24   | 12   |
|                                        | 200              | 16                         | 34   | 13   | 10   |
|                                        | 100              | 10                         | 26   | 8    | -2   |
|                                        | 50               | 5                          | 12   | 4    | 0    |

| EAC1-3d                              | 600              | 4                          | ND*  | ND   | ND   |
|                                        | 400              | 0                          | 45   | -10  | 85   |
|                                        | 300              | ND                         | 31   | 0    | 57   |
|                                        | 100              | ND                         | 10   | ND   | 20   |
|                                        | 50               | ND                         | -5   | ND   | 15   |

* ND, not done.

With normal blood high-density polymorphonuclear neutrophils (Table III), fluid-phase C4b, C3b, and C3c each produced dose-dependent inhibition of both EAC14b and EAC1-3b rosette formation, whereas fluid-phase C3d had no demonstrable effect. Since the molecular weight of C3d was 14-20% of the other fragments, the molar concentrations of C3d employed were five to seven times greater than for the other fragments. Parallel tests of lymphocytes demonstrated that this C3d fragment preparation was fully active in blocking lymphocyte CR1-dependent rosettes. EAC14b rosettes were more easily inhibited than were EAC1-3b rosettes, but in both cases the different fragments had the same relative inhibitory capacity: C3b > C4b > C3c. Thus, C4b inhibited EAC1-3b rosettes and C3c inhibited EAC14b rosettes, indicating that neutrophils contain a single CR1-type receptor that is specific for either C4b or the C3c region of C3b.

A somewhat different result was obtained with fractions containing less mature neutrophils (Table IV). EAC1-3b rosette formation was inhibited slightly by high concentrations of fluid-phase C3d, while fluid-phase C4b and C3c each produced less than half as much inhibition of EAC1-3b rosettes as did fluid-phase C3b. This suggested that C3b was bound by receptors specific for both the C3c and C3d regions. The binding of C3b to C3d region receptors (CR3) was confirmed by the observation that fluid-phase C3b, as well as C3d, caused significant inhibition of EAC1-3d rosette formation. EAC14b was bound only to C3c region receptors (CR1), because EAC14b rosettes were inhibited by fluid-
Expression of CR₁, CR₂, and Ia Antigens on Leukemic Myeloid Cells

| Cell source | Number of patients | Density layer | CR₁ | CR₂ | Ia Ag |
|-------------|--------------------|---------------|-----|-----|-------|
|             |                    | g/ml          | %   |     |       |
| CML         |                    |               |     |     |       |
| High CR₂ (>50%) | 7                  | 1.05-1.07     | 19  | 47  | 25    |
|             |                    | 1.08-1.09     | 49  | 66  | 2     |
|             |                    | 1.105         | 84  | 42  | 0     |
|             |                    | 1.12          | 86  | 44  | 0     |
| Low CR₂ (<25%)  | 9                  | 1.05-1.07     | 27  | 11  | 19    |
|             |                    | 1.08-1.09     | 53  | 15  | 1     |
|             |                    | 1.105         | 78  | 26  | 0     |
|             |                    | 1.12          | 64  | 6   | 0     |
| AML         | 5                  | 1.05-1.07     | 32  | 16  | 53    |
|             |                    | 1.08-1.09     | 47  | 25  | 10    |
|             |                    | 1.105         | 69  | 49  | 2     |
|             |                    | 1.12          | 47  | 40  | 3     |

Phase C₃c and C₃b, but not by C₃d, and because fluid-phase C₄b did not inhibit EAC₁-3d rosette formation. These results indicated that immature neutrophils contained CR₁- and CR₂-type receptors similar to those of B lymphocytes. Because of the dual reactivity of C₃b with both CR₁ and CR₂, EAC₁4b rosette formation was used instead of EAC₁-3b rosette formation as a specific marker of CR₁, and EAC₁-3d rosette formation was used as a specific marker of CR₂.

Sequential Expression of CR₁ and CR₂ at Different Stages of Neutrophil Maturation. The observation with blood and marrow that CR₂ was expressed more on low density immature neutrophils than on high density mature neutrophils, suggested that CR₂ was expressed during neutrophil maturation before the appearance of CR₁, and thus might be a marker of relative neutrophil immaturity. The support for this was provided by the finding that the low density immature neutrophils from 7 out of 16 patients with CML contained significantly more CR₂- than CR₁-bearing cells, whereas the high-density more mature neutrophils from these same seven patients contained more CR₁- than CR₂-bearing cells. The neutrophils from these seven CML patients were grouped together because they differed from those of other patients and normal marrow in that they contained a high proportion of CR₂-bearing cells (Table V). In these seven CML patients, the excess of CR₂-bearing cells were isolated in low density fractions along with the relatively less mature cells, whereas in the remaining patients and in normal marrow, the proportion of CR₂-bearing cells was less than the proportion of CR₁-bearing cells in all density fractions. All of these neutrophil preparations resembled normal blood neutrophils in that CR₂ was decreased on mature neutrophils in 1.12-g/ml fractions as compared to the relatively less mature neutrophils contained in low and intermediate density fractions. Thus, high density mature neutrophils consistently lost CR₂ and retained CR₁.
TABLE VI

Expression of CR\textsubscript{1} and CR\textsubscript{2} on Neutrophil Maturation Stages Isolated from Normal Bone Marrow

| Wright-Giemsa morphology | CR\textsubscript{1} | CR\textsubscript{2} |
|--------------------------|---------------------|---------------------|
| Myeloblast               | 1                   | 2                   |
| Promyelocyte             | 2                   | 3                   |
| Myelocyte                | 3                   | 18                  |
| Metamyelocyte            | 18                  | 21                  |
| Band form                | 29                  | 23                  |
| Polymorphonuclear        | 54                  | 35                  |

Since the presence of normal marrow CR\textsubscript{1}– CR\textsubscript{2} immature neutrophils could not be inferred from measurement of the overall proportions of cells bearing CR\textsubscript{1} and CR\textsubscript{2} in low density fractions, the C-receptor type of immature and mature neutrophils was separately evaluated by examining fixed smears of neutrophil rosettes stained with Wright-Giemsa (Table VI). An average of 18% of myelocytes expressed CR\textsubscript{2}, but only 3% of myelocytes expressed CR\textsubscript{1}. CR\textsubscript{1} appeared on 18% of metamyelocytes bearing CR\textsubscript{2}, and then the expression of both types of C receptors gradually increased with cell maturation until the low density polymorphonuclear stage. Finally, high density polymorphonuclear neutrophils primarily expressed only CR\textsubscript{1} and lacked CR\textsubscript{2}.

Independent Expression of Ia Antigens and C Receptors. Human Ia antigens were detected by immunofluorescence on immature myeloid cells isolated at densities ≤ 1.06 g/ml from normal bone marrow or leukemic blood (Tables I, V). Ia determinants were only detected on myeloid cells that had the appearance of myeloblasts and promyelocytes in phase-contrast microscopy, and this was supported by parallel examination of preparations after Wright-Giemsa staining. In all of the normal marrows and in most cases of leukemia, myeloid cells containing C receptors were isolated at higher densities than cells expressing Ia antigens. Occasionally, however, cell fractions isolated from leukemic blood at intermediate densities contained both Ia antigen-bearing cells and C-receptor-bearing cells. These were studied to determine if Ia determinants and CR\textsubscript{1} or CR\textsubscript{2} were expressed on the same cells. Double-marker studies of 800 Ia-bearing cells from three individuals with CML revealed that 97–99% of Ia-bearing cells lacked both CR\textsubscript{1} and CR\textsubscript{2}.

Discussion

The principal finding of the present study was that Ia antigens and two types of C receptors (CR\textsubscript{1} and CR\textsubscript{2}) were expressed on the membrane surfaces of human neutrophils in a distinct sequence during maturation. The two types of neutrophil C receptors had a similar specificity to those previously described on the surface of B lymphocytes (14). Ia antigens were only detected at the earliest stage of maturation before the appearance of either type C receptor. Thus, the sequence of appearance of these three surface markers defined four potential stages of neutrophil membrane maturation:
Ia \rightarrow \text{CR}_2 \rightarrow \text{CR}_1 \rightarrow \text{CR}_0

Since polymorphonuclear neutrophils are the predominant myeloid cell form in both normal bone marrow and blood from patients with CML, surface marker analysis of immature neutrophils required separation of immature and mature granulocytes. Several previous studies have shown that immature myeloid cells had a lower density than did mature granulocytes (15–18). The density gradient technique was particularly valuable both for isolating myeloblasts and for separating mature neutrophils from eosinophils. Whereas normal bone marrow contained only 0.1–2.0% myeloblasts, low density fractions isolated from marrow contained an average of 23% myeloblasts. Furthermore, low density fractions obtained from the blood of patients with CML were enriched from 50- to 300-fold in the proportion of myeloblasts.

The specificity and number of neutrophil C receptors was determined in the same way as was previously reported with B lymphocytes, erythrocytes, and eosinophils (14, 19). Low and intermediate density neutrophils differed from high density neutrophils in that variable proportions of the low density cells formed EAC1-3d rosettes, whereas high density neutrophils formed rosettes with EAC14b and EAC1-3b but not with EAC1-3d. This suggested that low density immature neutrophils might contain only \text{CR}_0 and high density mature neutrophils might lack \text{CR}_2 and contain only \text{CR}_1. For this reason, the C receptor specificity of neutrophils was separately analyzed in two different density fractions. The first was a 1.12-g/ml fraction containing only polymorphonuclear neutrophils that formed rosettes with EAC14b and EAC1-3b but not with EAC1-3d, and the other was a morphologically less mature 1.08-g/ml fraction that formed similar proportions of rosettes with all three types of EAC. The finding that EAC1-3d rosettes were inhibited by fluid-phase C3d and C3b, but not by fluid-phase C3c or C4b, indicated that low density neutrophils contained a C3d receptor that had similar specificity to the CR_2 of B lymphocytes (14). Polymorphs that did not form EAC1-3d rosettes, contained a single CR_1 type of receptor for EAC14b and EAC1-3b rosette formation, because complete cross-blocking by fluid-phase C4b, C3b, and C3c fragments was observed. Of these three fragments, C3b had the highest affinity for CR_1, followed next by C4b and then C3c. Low density neutrophils that contained both CR_0 and CR_2 differed slightly in the mechanism of EAC1-3b rosette formation from their high density counterparts. Fluid-phase C4b and C3c produced considerably less inhibition of EAC1-3b rosette formation than did fluid-phase C3b, and fluid-phase C3d produced slight inhibition of EAC1-3b rosette formation. This suggested that C3b was partially bound to CR_0, even though most of the C3b was bound to CR_1. The binding of C3b to C3d region receptors (\text{CR}_2) was further confirmed by the demonstration of strong inhibition of EAC1-3d rosettes by fluid-phase C3b. Thus, neutrophil C receptors had identical specificity to the analogous receptors of B lymphocytes, eosinophils, and erythrocytes (14, 19). CR_1 was specific for C4b, C3c, or the C3c region of C3b, and CR_2 was specific for C3d fragments or the C3d region of C3b.

In the present study only 17% of normal blood neutrophils were found to contain \text{CR}_2, and these were concentrated at the 1.08-g/ml density interface, whereas the majority of blood neutrophils had a higher density and contained
TABLE VII
Correlation of Surface Markers with Maturation Stage and Cell Density

| Density     | Myeloblast | Promyelocyte | Myelocyte | Metamyelocyte |
|-------------|------------|--------------|-----------|---------------|
| 1.05-1.06 g/ml | Ia⁺“CR⁺,”CR⁺⁻ | Ia⁺“CR⁺,”CR⁺⁻ | Ia⁺“CR⁺,”CR⁺⁻ | Ia⁺“CR⁺,”CR⁺⁻ |
| 1.06 g/ml   | Ia⁺“CR⁺,”CR⁺⁻ | Ia⁺“CR⁺,”CR⁺⁻ | Ia⁺“CR⁺,”CR⁺⁻ | Ia⁺“CR⁺,”CR⁺⁻ |
| 1.07 g/ml   | Ia⁺“CR⁺,”CR⁺⁻ | Ia⁺“CR⁺,”CR⁺⁻ | Ia⁺“CR⁺,”CR⁺⁻ | Ia⁺“CR⁺,”CR⁺⁻ |
| 1.08 g/ml   | Ia⁺“CR⁺,”CR⁺⁻ | Ia⁺“CR⁺,”CR⁺⁻ | Ia⁺“CR⁺,”CR⁺⁻ | Ia⁺“CR⁺,”CR⁺⁻ |
| 1.09-1.105 g/ml | Ia⁺“CR⁺,”CR⁺⁻ | Ia⁺“CR⁺,”CR⁺⁻ | Ia⁺“CR⁺,”CR⁺⁻ | Ia⁺“CR⁺,”CR⁺⁻ |
| 1.12 g/ml   | Ia⁺“CR⁺,”CR⁺⁻ | Ia⁺“CR⁺,”CR⁺⁻ | Ia⁺“CR⁺,”CR⁺⁻ | Ia⁺“CR⁺,”CR⁺⁻ |

Previously, neutrophils were believed to bear only CR₁ and to lack CR₂. Gigli and Nelson (2) had demonstrated inhibition of EAC1-3b phagocytosis by C3b inactivator, and more recent studies overlooked neutrophil CR₂ because low density polymorphs were excluded by the isolation procedure, or alternatively, a low proportion of EAC1-3d rosettes were ascribed to residual uncleaved C3b or C4b on the EAC1-3d (3, 4).

Parallel assays of density gradient fractions for surface markers and Wright-Giemsa stain morphology demonstrated a distinct sequence of appearance of surface markers during neutrophil maturation. Ia antigens were present on myeloblasts and rare promyelocytes, and double-marker assays demonstrated that Ia determinants were lost before the first appearance of CR₁ on 18% of myelocytes. The proportion of myeloid cells expressing CR₂ increased with maturation until the low density polymorphonuclear stage and then decreased as polymorph density increased from 1.09 to 1.12 g/ml. CR₁ appeared first along with CR₂ on 18% of metamyelocytes and was maximally expressed on CR₂-negative, high density polymorphonuclear neutrophils. The correlation of surface markers, maturation stage, and cell density is summarized in Table VII.

It is important to note that the appearance or disappearance of surface markers was not abrupt at the individual maturation stages defined by Wright-Giemsa morphology. Also, although there was some variation in the relative time-course of marker expression among different cell preparations, the overall sequence of marker expression was apparently always the same. For example, Ia determinants were usually not detectable on the cell surface after the promyelocyte stage, however, in some few patients with CML, rare myelocytes and metamyelocytes contained Ia determinants, while in some other CML patients, Ia determinants were only detectable on 20-40% of myeloblasts and were absent from the majority of myeloblasts and more mature myeloid cells. In particular, C receptor expression was not strictly correlated with morphologic maturation state. The proportion of normal marrow cell bearing CR₂ increased from 18% at the myelocyte stage to 35% at the polymorph stage, and thus it is unclear whether or not all neutrophils pass through a CR₂-positive stage at some time during their development. CR₂ expression may be a characteristic feature of a distinct subset of neutrophils, for example, those that make up the marginating pool of noncirculating neutrophils. Alternatively, CR₂ expression may be one of the terminal maturation events that immediately precedes the
release of cells into the periphery, so that the kinetics of release prevent the accumulation of CR₇-bearing cells in the marrow. This latter mechanism may also explain the finding that only half of marrow high density morphologically mature neutrophils contain CR₇, whereas nearly all of peripheral blood neutrophils bear CR₇.

Thus, although there was a defined order of appearance of surface markers during maturation, the sequence was sometimes shifted slightly either to the right or to the left relative to the sequence of morphological maturation, and all three surface markers examined were sometimes undetectable on a proportion of the myeloid cells at any one stage of maturation. Studies of cultured murine leukocytes have demonstrated that the expression of C receptors can be induced on myeloblasts with prednisolone and actinomycin D (20). Taken together, these findings suggest that the regulation of membrane differentiation may be independent from the events of nuclear and cytoplasmic maturation defined by standard morphologic criteria.

Since CR₁ and CR₂ are also present on B lymphocytes and macrophages, and these cell types and neutrophils are believed to originate from a common marrow stem cell, it is possible that these other cell types may have the same sequence of C-receptor appearance during maturation as do neutrophils. For example, B lymphocytes from normal blood, considered to be at a stage of relative maturity, are either CR₁⁺ CR₂⁺ or CR₁⁺ CR₂⁻. On the other hand, in tonsils, leukemic blood, and normal bone marrow, each believed to contain a proportion of more immature B cells, a significant proportion of lymphocytes are CR₁⁻ CR₂⁺ (3, 14). There is also similarity in the terminal phase of murine macrophage differentiation into activated cells, CR₁⁺ CR₂⁺ → CR₁⁺ CR₂⁻ (5). Thus, with granulocytes, monocytes, and B lymphocytes, there may be a similar sequence of C-receptor appearance during cell differentiation: CRₛ → CR₁, CRₛ → CR₂. The same sequence of C receptor appearance was also found in parallel studies of murine neutrophil maturation (5, 21, 22). The only difference in C-receptor expression between the two species was in the incidence of CRₛ expression on blood polymorphs. The vast majority of mouse blood polymorphs contained both CR₁ and CR₂, while by contrast, only 17% of human blood polymorphs contained both types of C receptors (5).

The functional significance of a CR₁⁺ CR₂⁻ profile on fully mature neutrophils and activated macrophages has been difficult to explain in relation to a plasma C₃b inactivator that renders EAC14b and EAC1-3b complexes unreactive with CR₁-bearing cells. Immune complexes in plasma fix C₄b and C₃b that are rapidly cleaved by the plasma C₃b inactivator into the respective c and d fragments (11, 23). The C₄b cleavage products, C₄c and C₄d, are both believed to be unreactive with C receptors (24), while the complex-bound C₃b cleavage product, C₃d, only has activity with CR₂. In vivo however, C₄b sites on immune complexes may be partially protected from C₃b inactivator cleavage by formation either of a short-lived complex with C₂ (11) or with the low molecular weight C₄b-binding protein (25). This may help preserve C₄b sites long enough to allow attachment of immune complexes to CR₁-bearing phagocytes. There is similar complexity in the in vivo reactions involving immune complex-bound
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C3b. It is well known that when the immune complexes are sheep erythrocytes (EAC), the cell-bound C3b cleavage product, the C3d fragment, has little or no opsonic activity (2, 26, 27). However, under normal circumstances in vivo, C3-coated bacteria and yeast are the more usual fare of phagocytes, and erythrocyte target cells are more likely to be handled instead by lysis with natural antibodies and complement. Many strains of bacteria and yeast spontaneously trigger the alternative pathway of C activation and as a result become coated with C3b that is subsequently cleaved by C3b inactivator. However, in contrast to sheep cell membranes, Stossel et al. (26) have shown that bacterial cell wall-derived lipopolysaccharide-coated paraffin oil particles retain an opsonically active form of C3 despite prolonged incubation with whole serum. Furthermore, Fearon and Austen (28) have demonstrated that yeast cell wall-derived zymosan surfaces differ from sheep erythrocyte membranes in that alternative pathway-derived C3b is bound in such a way that it is relatively resistant to cleavage by C3b inactivator. Thus, with both bacteria and yeast, C3b may be an effective CR, reactive opsonin despite the presence of C3b inactivator, and mature phagocytes may only need to contain CR type C receptors for effective clearance of these microbes.

Summary

Ia antigens and two different types of complement (C) receptors appeared on membrane surfaces in a distinct sequence during the maturation of human neutrophils. Taking advantage of the finding that neutrophil cell density increased with maturation, density gradient centrifugation was used to separate neutrophils into fractions that were greatly enriched in cells representing individual stages of differentiation. Myeloblasts, the earliest cells recognized in the myeloid series of both normal and myelogenous leukemic individuals, expressed Ia determinants, whereas Ia determinants were absent or diminished on the majority of promyelocytes and completely undetectable on more mature granulocytes. Double marker studies demonstrated that Ia determinants were lost from the membrane of developing myeloid cells before the appearance of any type of C receptor. In the next phase of maturation defined by surface markers, neutrophils acquired a CR type C receptor (C3d receptor) that was similar in specificity to CR2 of B lymphocytes. This stage of maturation approximately corresponded to the myelocyte-metamyelocyte stage defined by standard morphologic criteria, and preceded the third stage of surface marker maturation when developing neutrophils began to express CR1-type C receptors (immune adherence, C4b-C3b receptors) in addition to CR2. In the final stage of surface marker-defined maturation, CR2 was lost from high density polymorphonuclear neutrophils and CR1 was maximally expressed. Normal blood polymorphonuclear neutrophils contained only 17% of CR2-bearing cells and these were shown to be of lower density than the majority of neutrophils that expressed only CR1. There was some variation in the correlation of surface marker expression and maturation stage defined by morphologic criteria, but in all cases the sequence of marker appearance was the same:

Ia → CR2 → CR1CR2 → CR1
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