Distribution of Complement Receptor Subtypes in Non-Hodgkin’s Lymphomas of B-Cell Origin

By Jeffrey Cossman and Elaine S. Jaffe

Surface receptors specific for either the C4b (CR1) or C3d (CR2) component of complement were examined on the neoplastic cells from 30 cases of non-Hodgkin’s lymphoma of B-cell origin and on cells derived from 9 normal lymphoid tissues. Lymphocyte suspensions from non-neoplastic peripheral blood, tonsils, and lymph node contained three categories of complement receptor lymphocytes (CRL): cells with receptors for both C4b and C3d (CR1 + , CR2 + ); cells with receptors for C4b but not C3d (CR1 + , CR2 - ), and cells with receptors for C3d but not C4b (CR1 - , CR2 + ). The mean of the proportion of total CRL expressing receptors only for C3d (CR1 - , CR2 + ) was 0.35 for non-neoplastic tissues and 0.28 for malignant lymphomas.

The B-cell lineage of normal and neoplastic human lymphocytes is determined by the detection of endogenously synthesized surface immunoglobulin (slg) and/or intracytoplasmic immunoglobulin (clg). Other markers that are associated with B cells include complement receptors (CR), Fc receptors (FcR) and Ia antigens, and are present on most, but not all, normal B cells.1,2 The extremes of B-cell differentiation, i.e., the pre-B-cell and plasma cell stages, can be distinguished by the array of immunologic markers expressed; pre-B-cells are usually devoid of slg but contain small amounts of clgM,3 whereas plasma cells contain abundant clg but may lack slg.4 However, relatively few markers are available to distinguish stages of either B-cell ontogeny or B-cell differentiation beyond the pre-B-cell stage.

In the present study, we have explored the expression of surface markers on normal and neoplastic B cells in order to determine whether these markers might be useful in distinguishing cells at various levels of differentiation. We have focused particular attention on the expression of subtypes of complement receptors on B cells. Human B lymphocytes possess at least two distinct complement receptors, one for C3d (CR2) and another that binds both C3b and C4b (CR1).1,2 It has been previously reported that during neutrophil (differentiation, the appearance of CR specific for the C3d component of complement (CR2) precedes the acquisition of receptors for C3b (CR1), and only the C3d receptor (CR2) is expressed on mature neutrophils.5 Although both CR1 and CR2 are present on normal B cells,1,2 it is unknown whether a similar sequential acquisition and loss of these receptors occurs during differentiation. Non-Hodgkin’s lymphomas of B-cell type frequently express CR, which are detectable in both cell suspensions and on frozen tissue sections.6-8 In addition, these lymphomas are heterogeneous with respect to their expression of subtypes of complement receptors.8 It has been previously demonstrated that the neoplastic nodules of follicular center cell (FCC) origin contain cells with the same distribution of CR subtypes as non-neoplastic reactive germinal centers.9 The present investigation expands on this observation by comparing the distribution of CR subtypes on the neoplastic cells of a selection of B-cell lymphomas that most consistently express CR, namely well differentiated lymphocytic lymphoma (WDL), intermediate differentiated lymphocytic lymphoma (IDL) and lymphomas of FCC origin (nodular poorly differentiated lymphocytic and mixed cell types).6-9 It is the purpose of this study to quantify the distribution of CR subtypes in B-cell lymphomas and investigate whether CR subtype expression distinguishes between the categories of these lymphomas as classified by morphological and clinical features.

MATERIALS AND METHODS

Cells and Tissues

Viable cell suspensions were prepared immediately after biopsy of 9 non-neoplastic and 30 lymphomatous tissues. The lymphomas...
include 10 cases of CLL or WDL, 6 cases of IDL, and 14 cases of follicular center cell type (10 poorly differentiated lymphocytic type and 4 mixed cell type; 12 were nodular; 2 were diffuse). Peripheral blood lymphocytes were depleted of monocytes by phagocytosis with carboxylin and separated on a Ficoll-Hypaque gradient. In most instances, cell suspensions were viable frozen in 10% dimethyl sulfoxide (DMSO) over liquid N2 and stored for use at a later date. Viability was always greater than 80% following thawing, and surface marker values were unaffected.

For frozen sections, tissue blocks were embedded in O.C.T. medium (Lab-Tek Products, Naperville, Ill.), snap frozen in isopentane and dry ice, and stored at −70°C. Diagnoses were made on routinely prepared, B-5-fixed tissue according to a modification of the Rappaport classification. The Raji cell line was a gift of Dr. Alan S. Rabson, National Cancer Institute, National Institutes of Health, Bethesda, Md., and the Daudi cell line was a gift of Dr. A. N. Theofilopoulos, Scripps Clinic and Research Foundation, La Jolla, Calif.

Detection of Complement Receptors

IgM antibody sensitized sheep erythrocytes (EA) were coated with either C4b (EAC4b) or C3d (EAC3d) according to the method of Ross et al. EA were sensitized with a subagglutinating concentration of the IgM fraction of rabbit anti-sheep erythrocyte antibody (Cordis Laboratories, Miami, Fla.). EA were unreactive with lymphocytes and served as a negative control for all cases. EAC4b was prepared by treatment of EA with an excess of human C1, followed by incubation with heat-inactivated (56°C, 30 min) normal human serum as the source of C4. EAC3d was prepared by incubation of EA with mouse (AKR) serum (1:20) in gelatin veronal-buffered saline containing calcium and magnesium (GVB+) at 37°C for 45 min. The cells were washed repeatedly in GVB containing ethylene diamine tetraacetate acid (EDTA) (GVB-EDTA) and incubated in GVB-EDTA for 30 min at 37°C. A final incubation with mouse serum (1:2.5) in GVB-EDTA at 37°C for 45 min was performed to assure complete cleavage of cell-bound C3 to the C3d form by the serum enzyme C3b inactivator. The cells were finally washed and resuspended in GVB++. For dual label assays, sheep erythrocytes were fluoresceinated with fluorescein-isothiocyanate (FITC) prior to sensitization and complement fixation. No nonfluorescent erythrocytes were detected in these samples.

Complement-coated fluorescent bacteria were prepared according to the method of Gormus et al. Salmonella typhimurium were treated with either FITC or tetra-rhodamine isothiocyanate (TRITC). The antibody for sensitization was prepared by subcutaneous 1-ml injections of rabbits (New Zealand Whites) with heat-killed S. typhimurium (10^6 bacteria/ml). Serum was obtained at 1–2 wk after initial injection. Heat-killed bacteria were sensitized with subagglutinating concentrations of this serum (BA) and subsequently incubated at 4°C for 40 min to make BAC. BA or BA incubated with heat-inactivated serum failed to bind to lymphocytes in all cases.

For rosette assays, equal volumes of lymphocytes (4 x 10^7/ml) in RPMI 1640 medium (Microbiological Associates, Walkersville, Md.) and either EAC (10^7/ml) or BAC (10^7/ml) were incubated together at 37°C for 30 min. Lymphocytes with three or more EAC or BAC bound to their surfaces were considered positive.

Frozen Section Rosette Assay

Air-dried frozen sections were layered with either EAC4b or EAC3d, incubated, fixed, and stained as previously described.

Non-specific Esterase Activity

In order to determine whether rosetting cells were nonlymphoid, i.e., mononuclear phagocytes, cytocentrifuge preparations of rosetted cells were stained for non-specific esterase by the a-naphthyl butyrate esterase method. In all instances, fewer than 10% of rosetted cells were esterase positive, indicating that monocytes and/or histiocytes were not a significant contaminant of either peripheral blood mononuclear or tissue suspensions.

RESULTS

Specificity of EAC and BAC

The specificity of the indicator particles for CR1 and CR2 was determined by their ability to bind to human erythrocytes, Raji, or Daudi cells (Table 1). Human erythrocytes bear receptors for C3b and C4b, probably via a single receptor (CR1+), but fail to bind C3d (CR2−). Raji and Daudi cells possess avid receptors for C3d and possibly C3b.

BAC readily adhered to both Raji and Daudi cells but failed to adhere to human erythrocytes (Table 1). Therefore, BAC bear C3 in the form of C3d but not C3b. EAC3d (prepared with mouse serum) demonstrated the identical specificity as BAC (Table 1). EAC4b adhered to human erythrocytes but not Raji and Daudi cells, and thus do not have surface-fixed complement receptors.

Table 1. Complement Component Specificity of EAC and BAC

<table>
<thead>
<tr>
<th>Indicator Particle</th>
<th>Human Erythrocytes</th>
<th>Raj</th>
<th>Daudi</th>
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<tr>
<td></td>
<td>Rosette Formation (%)*</td>
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<td></td>
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<tr>
<td>EAC4b (hu)</td>
<td>62 (3.5)</td>
<td>2 (0.4)</td>
<td>1 (0.3)</td>
</tr>
<tr>
<td>EAC3d (mo)</td>
<td>0 (0)</td>
<td>100 (0.5)</td>
<td>86 (1.8)</td>
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<td>BAC3d (mo)</td>
<td>0 (0)</td>
<td>99 (0.6)</td>
<td>93 (2.6)</td>
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*Mean of four separate preparations (± SE).
Table 2. Complement Receptor Subtypes of Normal Lymphocytes

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Single Label</th>
<th>EAC4b- EAC3d+</th>
<th>EAC4b+ EAC3d-</th>
<th>EAC4b- EAC3d+</th>
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<tr>
<td></td>
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<td></td>
<td></td>
<td>Total CRL</td>
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<td></td>
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<td>0.35 ± 0.06</td>
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C3d. In addition, EAC4b agglutinated when incubated with anti-human C4 but not anti-human C3 (Cappel Laboratories, Downington, Pa.).

**Complement Receptor Subtypes of Normal Lymphocytes—Cell Suspensions**

Lymphocyte populations from all nine non-neoplastic tissues demonstrated rosette formation with both EAC4b and EAC3d using a single label assay (Table 2). The percentage of CR2+ cells tended to slightly exceed the percentage of CR1+ cells. The dual label technique revealed three separate categories of CR+ cells (Fig. 1). The majority of CRL formed rosettes simultaneously with both EAC4b and EAC3d (CR1+, CR2+). Approximately one-third of CRL bound EAC3d exclusively (CR1-, CR2+), and a small percentage demonstrated only CR1 receptors. Because of the relatively high proportion of (CR1-, CR2+) cells, a convenient means of displaying the distribution of CR subtypes is by the ratio of (CR1-, CR2+) cells: total CRL. The mean of this ratio for non-neoplastic lymphoid tissues was 0.39 and for normal peripheral blood lymphocytes it was 0.31 with an overall mean (±SE) for non-neoplastic tissues of 0.35 ± 0.06. In all instances, the percentage of nonspecific esterase-positive cells was less than 2%.

![Fig. 1. Normal human peripheral blood lymphocytes simultaneously rosetted with EAC4b (FITC) and EAC3d. (A) Three rosetted lymphocytes are present. (B) Same field as (A) but illuminated and filtered for FITC to identify EAC4b (FITC). The lymphocyte in the upper left corner of the field (arrow in A and B) binds both EAC4b (FITC) and EAC3d, whereas the lymphocyte immediately below binds only the nonfluorescent EAC3d. The lymphocyte in the right side of the field binds only EAC4b-FITC. Thus, three complement receptor phenotypes are present in this field: CR1+, CR2+, CR1-, CR2+; and CR1+, CR2-. (Original magnification X450.)](image-url)
Due to the large fraction of CR1−, CR2+ cells, the question arose whether all of these cells were, in fact, B cells. A dual label assay simultaneously detecting slg and CR2 (using BAC3d) demonstrated that all CR2+ cells were also slg+ (Table 3).

**Complement Receptor Subtypes of Neoplastic Lymphocytes—Cell Suspensions**

Both CR1 and CR2 were detected on the neoplastic cells from all 30 cases and this was documented morphologically by examination of cytocentrifuge preparations of rosetted cells. The percentages of rosette formation are presented in Table 4 and represent the proportion of total mononuclear cells that formed rosettes. The single label assay demonstrated that fewer cells were CR1+ than were CR2+ and this difference was particularly evident for CLL-WDL and IDL. The dual label rosette assay further elucidates the distribution of complement receptor subtypes. Few cells formed rosettes with EAC4b only (CR1+, CR2−) for all histologic subtypes. The proportion of total complement receptor cells that bound only EAC3d (CR1−, CR2+:total CRL) was 0.65 for CLL-WDL, and 0.59 for IDL, whereas the FCC group was significantly lower (0.28) (p<0.025 against either WDL-CLL or IDL). No significant difference was demonstrated between CLL-WDL and IDL.

**Histologic Distribution of Complement Receptor Subtypes**

Frozen tissue sections of normal tissues (tonsils, spleen, and lymph node) exhibited adherence of EAC3d to lymphoid follicles (Fig. 2A). EAC4b bound not only to follicles but to cells within the red pulp as well (Fig. 2B). The nodules of nodular lymphomas exhibited adherence of both EAC3d and EAC4b, although in some instances EAC4b attached predominantly to the periphery of the nodules. EAC4b, but not EAC3d, was present in interfollicular areas, particularly in the red pulp of spleens involved by lymphoma. Frozen sections of a lymph node replaced by WDL bound EAC3d, but not EAC4b.

**DISCUSSION**

The present study demonstrates the following: (1) normal and neoplastic B lymphocytes bear receptors for both EAC4b and EAC3d; (2) some normal B cells bind both EAC types, whereas others bind only one; (3) complement receptor-positive B-cell lymphomas of different histologic subtypes differ in their distribution of complement receptor subtypes; and (4) both EAC4b and EAC3d bind to cells in normal lymphoid follicles and neoplastic nodules of lymphoma, whereas adherence of EAC4b predominates between follicles and nodules.

From the data derived from dual label studies, at least three distinct subpopulations of CRL exist (CR1+, CR2−; CR1−, CR2+; CR1+, CR2+). A previous study by Ross et al., using EAC reagents, suggested that all peripheral blood possess CR1.2 However, in that investigation, a dual label assay for the simultaneous detection of CR1 and CR2 receptors was not used. In addition, monocytes were not depleted from peripheral blood mononuclear cells2 and, since cells of the macrophage-monocyte series possess predominately CR1 but not CR2 receptors,14 this might account for the elevated percentages of cells bearing CR1. Although a dual label rosette assay may have limitations, such as competition for membrane attachment by relatively large particles and the suboptimal sensitivity inherent in rosette assays, the present study does demonstrate that CRL are heterogeneous in their ability to bind fixed complement components. Competition for receptor sites between the two types of EAC did not appear to affect the detection of CRL, since the total percentages of either CR1 cells or CR2 cells are approximately the same in both the single and dual label assays (Tables 2 and 4). Investigations to
Fig. 2. Frozen sections of normal human spleen incubated with EAC4b (A) and EAC3d (B). EAC4b attached to both the follicles (B-cell zones and interfollicular areas, while EAC3d adhered only to follicles. (Original magnification X72, darkfield.)
COMPLEMENT RECEPTORS IN B-CELL LYMPHOMAS

determine whether the failure to form rosettes with a specific EAC reagent is due to an absolute lack of the required receptor or to a subthreshold number, affinity, or avidity of receptors are currently being undertaken in our laboratory.

The three groups of B-cell neoplasms in this study differ in both morphology and clinical behavior. Intermediately differentiated lymphomas are composed of an admixture of small round lymphocytes like those of WDL and small cleaved follicular center cells similar to those of PDL and are presumably derived from cells of the lymphoid cuff. The "intermediate" nature of this lymphoma may also be reflected in the proportion of CRL that are CR1−, CR2+ (0.59), which falls between that of WDL-CLL (0.65) and FCC (0.28).

It has been suggested that IDL may be closely related to diffuse centrocytic lymphoma; however, the predominance of CR2 in IDL contrasts with the findings of Tolksdorf et al. in their study of diffuse centrocytic lymphoma in which the percentage of cells binding EAC3b was slightly higher than that binding EAC3d. One explanation for this discrepancy might be that EAC3b can potentially bind to cells with avid CR2, since C3d is present in the C3b molecule. This could then result in falsely elevated values for CR1. Alternatively, diffuse centrocytic lymphoma may be the diffuse counterpart of nodular poorly differentiated lymphocytic lymphoma (small cleaved cell, diffuse) which, in our hands, had nearly equal percentages of CR1 and CR2.

One implication of our study is that CR subtypes may be useful as differentiation markers, since the cells of non-Hodgkin’s lymphomas may be arrested or frozen at a particular stage of differentiation. However, in all cases, cells of all three CR phenotypes were detected, suggesting heterogeneity within the neoplastic cell population. This finding would support the theory of Taylor, which proposes that a broad range of B-cell differentiation may exist within any given human B-cell lymphoma. Conversely, flow cytometry analysis has demonstrated homogeneous densities of slg in CLL, suggesting a restricted range of differentiation. Murine lymphomas appear to express heterogeneous densities of cell surface markers; however, this heterogeneity may be exaggerated by variations in cell volume or cell cycle. The distribution of slg and CR densities on the cells from human lymphomas using flow cytometry is currently under investigation.

The pattern of binding of EAC3d to frozen sections of both normal lymphoid tissue and nodular lymphomas is similar to that previously reported and appears to be due to the specificity of EAC used in previous studies. We have found that routinely prepared EAC, using mouse serum as the complement source, such as that employed in previous studies, is nearly always immune adherence negative and thus specific for C3d. However, occasional preparations do adhere to human erythrocytes, and thus the specificity of routinely prepared EAC for C3d cannot be assumed and must be checked by the immune adherence assay. An additional complication in the preparation of EAC3d has recently been described. EAC-mo, as prepared in the present study, may contain an incompletely cleaved intermediate form of C3b known as C3bi, which probably does not bind to CR1 but may bind to C3d receptors or to a newly described third complement receptor, C3bi receptor. Although the C3bi receptor has been described on neutrophils and monocytes, its existence on either murine or human lymphocytes is, at present, unclear. If lymphocytes are shown to possess receptors for C3bi, then preparations of EAC3d for future studies should be incubated with proteolytic enzymes such as trypsin to assure complete cleavage to C3d. The adherence of EAC4b to normal lymphoid follicles, nodules of lymphoma, and inter-nodal areas was similar to that previously reported using EAC3b (which also binds to the same receptor as EAC4b). It is possible that EAC4b binds to interfollicular B lymphocytes. However, the possibility that EAC4b or EAC3b might have bound to interfollicular histiocytes or reticulum cells cannot be excluded, since these cells presumably possess CR1.

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