Human Lymphocyte Complement Receptors

QUANTITATIVE REQUIREMENTS FOR C3 OF NORMAL AND CHRONIC LYMPHOCYTIC LEUKEMIA LYMPHOCYTES

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ABSTRACT Erythrocytes coated with varying amounts of human complement were used to detect lymphocytes with complement receptors from normal subjects and patients with chronic lymphocytic leukemia. The relationship between the percentage of lymphocytes rosetting and the quantity of C3 present on complement-coated erythrocytes were studied. Small quantities of C3 (<5 fg/erythrocyte) caused maximal rosetting of normal lymphocytes. Maximal rosetting with chronic lymphocytic leukemia lymphocytes was not reached until much greater amounts of C3 were used to coat the erythrocytes. This difference in sensitivity to erythrocyte-bound complement was not due to an increased fraction of complement receptor-bearing cells in the leukemic patients. This loss of sensitivity of the chronic lymphocytic leukemia lymphocyte for complement may play a role in the immune deficiency present in this disease.

INTRODUCTION

Several types of human cells have receptors for the third component of complement (C3 (1, 2). Complement receptors on granulocytes, monocytes, and macrophages function in processes leading to phagocytosis (3–5). The role of complement receptors on a subpopulation of human lymphocytes is less clear, but evidence is accumulating that these receptors are involved in some aspects of immune recognition (6, 7). Lymphocytes with complement receptors (CRL)[1] have been considered bone marrow derived-(B) lymphocytes and serve a recognition function for the antibody-producing arm of the immune system (8–10). CRL have receptors for several complement constituents, including C3b (the hemolytically active fragment of C3) and C3d (a degradation product of C3b produced by the action of the plasma enzyme, C3b inactivator) (11, 12). There are also receptors for the fourth component of complement (12). In such studies, complement sources from several species prepared in varying ways have been used to coat indicator erythrocytes for the rosetting procedures. However, the quantitative requirements for C3 of human lymphocyte receptors have not been extensively detailed.

To study lymphocyte receptors for human complement, we coated human erythrocytes with complement by reacting them with anti-I antibody and whole human serum (13). We then used a quantitative anti-C3 consumption assay to measure the amount of the third component of complement added to these cells (14). By varying the concentration of serum present during the erythrocyte preparation procedure, it was possible to obtain erythrocytes coated with C3, predominantly in the form of C3b. In this paper we describe the quantitative C3 requirements for the rosetting of complement-coated erythrocytes with CRL from normal subjects and patients with chronic lymphocytic leukemia (CLL).

METHODS

Preparation of complement-coated human erythrocytes. Methods of complement-coating of human erythrocytes by anti-I antibody and normal human serum, and the means of detecting C3 bound to these cells have previously been described and will be briefly reviewed here (13–15). To determine the quantitative requirements for human C3 in rosetting with complement-reactive lymphocytes, we chose to investigate the consequences of complement activation in whole serum rather than that produced by fractions of purified human complement components. We did so to mimic more closely the natural situation, inasmuch as it appears that the reactions occurring in normal serum under such circumstances may differ from those occurring when purified components are used (16).

[1] *Abbreviations used in this paper*: CLL, chronic lymphocytic leukemia; CRL, complement-reactive lymphocytes; EC, complement-coated erythrocytes; VBS, veronal-buffered saline.

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Whole blood from normal, type 0 subjects was obtained by venipuncture and placed in Alsever's solution (17) until used. Erythrocytes were then washed in isotonic veronal-buffered saline with 1% gelatin (VBS) (17, 18) and resuspended at a concentration of 4.4×10^8 cells/milliliter in VBS before use. Type-compatible serum obtained from normal donors was either used immediately or stored at −90 C until used. Anti-I antiserum was obtained as previously described (13) (kindly provided by Dr. W. F. Rosse). A reaction mixture containing 1 part erythrocytes, 1 part anti-I diluted 1:40 with VBS, and 1 part serum at dilutions of from 1:16 to 1:256 in VBS were incubated at 4°C for 30 min. The reaction mixture was then warmed slowly over 5 min at 37°C. 10 vol of 37° VBS were added immediately, and the cells were centrifuged. The cells were then washed four additional times with 37°C VBS. A portion of the erythrocytes was resuspended in VBS to a concentration of 2.2×10^8/ml for determination of cell-bound C3, and the remainder diluted to a concentration of 1×10^7/ml for reaction with lymphocytes. This complement-coating procedure in dilute serum with a brief incubation at 37° C leads to the coating of erythrocytes with C3, predominantly in the form of C3b. This occurs because the cleavage of C3b to C3d by C3b inactivator requires higher serum concentrations or prolonged incubations at 37°C (14, 19). The cold-reactive antibody is entirely removed by the 37°C washing procedure such that complement-coated erythrocytes (EC) are coated with complement components only (13, 14).

Quantitation of membrane-bound C3. Membrane-bound C3 was measured by a previously described modification of the method of Borsos and Leonard (14, 20). Erythrocyte C3 was quantitated by absorption of a known amount of rabbit anti-human C3 during incubation with erythrocytes. The amount of anti-C3 remaining was measured by Lysis of C3-coated sheep erythrocytes with an excess of guinea pig serum as a source of complement. Human C3 was purified by a modification of the method of Nilsson and Muller-Eberhard (21). An euglobulin precipitate of fresh human serum was applied to a DEAE column equilibrated with 0.03 M phosphate buffer, pH 8.1, and eluted with a gradient from 0.03 M phosphate buffer, pH 8.1, to 0.2 M NaH_2PO_4. The C3-containing fractions detected by immunoprecipitation were applied in a batch technique to hydroxyapatite which had been equilibrated with 8 mmho phosphate buffer, pH 7.9; the hydroxyapatite was then washed with 12 and 13 mmho phosphate buffer, pH 7.9, and the C3-containing material was eluted with 14.5 mmho phosphate buffer. The C3-containing supernatant fluid was then concentrated by the method of Alper and Rosen (22). The purified human C3 produced a single band on microimmunoelectrophoresis against anti-whole human serum. (Meloy Laboratories, Inc., Springfield, V.) A single protein band was produced on polyacrylamide-gel disc electrophoresis. Anti-C3 antiserum was obtained by injecting rabbits with 0.75–2.0 mg of purified C3 emulsified in Freund's complete adjuvant. The rabbits were immunized three times over a 6-wk period, and the antisera used for this study were harvested 2 wk after the third immunization. The antisera were absorbed with bis-diazotized benzidine-aggregated IgG and human erythrocytes. When fresh human serum was examined by microimmunoelectrophoresis against these antisera, only a β_{1c} immunoprecipitin was obtained. When human serum, incubated at 4°C for 2 wk was similarly examined, β_{1a} and α_{2d} immunoprecipitins were produced. When partially aged serum was examined by this antiserum, B_{1c}, β_{1a}, and α_{2d} arcs were present. C3-coated sheep cells were also prepared as previously described (13–15).

Duplicate 0.1-ml serial three-fold dilutions of complement-coated or control human erythrocytes were mixed with 0.1 ml of anti-C3 for 30 min. 0.1 ml of C3-coated sheep cells was then added, without centrifugation and was allowed to react for 30 min, followed by the addition of 0.2 ml of guinea pig serum diluted 1:40 with VBS. After a final 30-min incubation, 5 ml of VBS was added, the mixture was centrifuged, and the percentage of sheep cells lysed was determined by spectrophotometric measurement of free hemoglobin at 412 nm. Negligible quantities of free hemoglobin were detected when color controls containing human EC, anti-C3, and guinea pig complement were incubated and assayed without sheep erythrocytes. The degree of absorption of anti-C3 antibody by C3-coated human cells was measured by diminution of lysis of the C3-coated sheep cells. The diminution in lysis was compared to that engendered by graded amounts of fluid phase C3. Due to possible differences between antigens present in membrane-bound C3 and those in native C3, these measurements of membrane-bound C3 may be relative and not absolute. Control human erythrocytes, which had been prepared without human serum or without anti-I antibody, did not diminish the lysis of C3-coated sheep cells by anti-C3. EC3 does not consume the unrelated antibody anti-human-IgG, as measured by inhibition of lysis of sheep erythrocytes coated with human IgG (23).

Preparation and quantitation of lymphocyte EC rosettes. Lymphocytes from normal subjects and from patients with chronic lymphocytic leukemia were prepared from heparinized whole blood. Erythrocytes were sedimented by the addition of 1/5 vol of Plasmagel (HTI Corp., Buffalo, N. Y.) and the resultant leukocyte-rich plasma was incubated at 37°C for 15 min with carbonyl iron powder (24) (GAF Corp., New York). The phagocytic cells were then removed by passing the iron-plasma mixture through a magnetic field. The leukocyte-rich plasma was centrifuged for 20 min at 800 *g* on a Ficoll-Hypaque gradient (Ficoll, Pharmacia Fine Chemical, Div. of Pharmacia, Inc., Piscataway, N. J., Hypaque, Winthrop Laboratories, N.Y.) to obtain a purified lymphocyte preparation (25). The interface containing >95% small lymphocytes was harvested and washed twice with isotonic phosphate buffered saline. The lymphocytes were resuspended in Media 199 to a concentration of 2.0×10^6/ml. Equal volumes (0.4 ml) of lymphocytes and C3-coated erythrocyte suspension were mixed in plastic tubes and incubated with shaking for 30 min at 37°C. Control incubations using uncoated human type O erythrocytes or erythrocytes prepared in the absence of serum or antibody were performed simultaneously. The erythrocyte-lymphocyte suspension was then gently mixed with 50 μl of methylene blue and placed in a leukocyte counting chamber. Duplicate reaction mixtures were prepared and a total of 400 lymphocytes were counted in each of the reaction mixtures to determine the percentage of lymphocytes forming rosettes (CRL). A lymphocyte rosette was defined as an identifiable single lymphocyte with three or more erythrocytes attached.

Lymphocyte characteristics. Ancillary data of the five patients with CLL and six control subjects studied are shown in Table I. Peripheral blood leukocyte number and differential leukocyte counts were done by routine clinical methods. Surface characteristics of the purified lymphocyte preparations from these individuals are also shown. The percentage of lymphocytes with detectable surface immunoglobulins was measured by previously described fluorescent microscopy techniques (24). Fluorescein-conjugated rabbit anti-human polyvalent immunoglobulin was used as antisera for this purpose. The fraction of lymphocytes forming rosettes with sheep erythrocytes was determined by previously described methods (26).

RESULTS

When lymphocytes from CLL patients or normal subjects were incubated with control erythrocytes, prepared either by reaction with antibody in the absence of serum or serum in the absence of antibody, no rosettes were observed. When the lymphocytes were incubated with erythrocytes coated with increasing amounts of C3, a dose-response relationship was obtained between the amount of C3 detected on the erythrocytes and the percentage of lymphocytes which formed rosettes. The results of such an experiment are shown in Fig. 1 for lymphocytes from a patient with chronic lymphocytic leukemia and lymphocytes from a normal subject. In this experiment the normal CRL reached maximal rosetting with small amounts of C3 present on erythrocytes. On the other hand, the CLL complement-reactive lymphocytes required large amounts of C3 to reach maximal rosetting. This difference in sensitivity between CLL and normal lymphocytes could be detected despite the similar percentage of CRL exhibited by this particular CLL patient and normal subject.

To examine further the C3 requirement for rosetting of normal and CLL lymphocytes, lymphocytes from six normal subjects and five patients with CLL were studied for reaction with erythrocytes coated with varying amounts of C3. The maximal percentage of CRL detected varied from 10 to 19% for the normal subjects from 21 to 70% for the lymphocytes of the CLL patients. The lymphocyte characteristics of these individuals are shown in Table I. To compare results from individuals with different maximal numbers of lymphocytes forming rosettes, the C3-coated erythrocyte rosetting data were expressed as the percentage of the maximal fraction of lymphocytes from that individual which would form rosettes with the EC tested. The percentage of maximal rosetting was determined by dividing the fraction of lymphocytes which form rosettes with a particular sample of EC by the highest fraction of rosette-forming cells detected in that lymphocyte sample by optimally coated erythrocytes. Percentage of maximal rosetting = fraction of lymphocytes forming rosettes at a given complement concentration/fraction of lymphocytes forming rosettes at an optimal complement concentration × 100. Optimal concentration of complement was the concentration resulting in the greatest number of lymphocytes forming rosettes for a sample from a given patient. The results of C3 rosette determinations using lymphocytes from six normal individuals and five CLL patients are shown in Fig. 2. As was suggested by the comparison in Fig. 1, cumulative results in Fig. 2 verify that considerably less C3 is necessary to induce rosetting with normal CRL than with CLL complement-reactive lymphocytes. When assayed with C3-coated erythrocytes, maximal rosetting of normal CRL occurred at levels of C3 of <5 fg per cell, whereas six to eight times as much surface-bound C3 was necessary to induce maximal rosetting of CLL CRL.

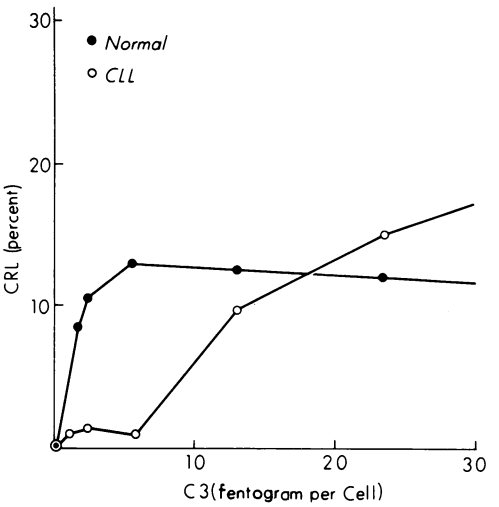

FIGURE 1 The relationship between the amount of cell-bound C3 and the percentage of lymphocytes forming rosettes with these erythrocytes is shown. (●), data of lymphocytes from normal subject 6; (○), lymphocytes from CLL patient E. Each point represents the percentage of lymphocytes forming rosettes at a given concentration of complement and is the average of duplicate membrane-bound C3 measurements and duplicate percent lymphocyte determinations.

TABLE I
Lymphocyte Characteristics of CLL Patients and Normal Subjects

	Peripheral blood		Purified lymphocytes		
CLL	Total leukocytes/cm	Lympho-cytes	Ig+	EC3 rosettes (maximal)	Sheep erythrocytes rosettes
		%		%	
A	45,900	93	86	57	2
B	87,700	95	90	21	1
C	22,200	94	87	69	3
D	17,100	90	88	57	4
E	24,800	85	93	23	2
Normal					
1	6,500	35	20	15	50
2	5,800	27	20	19	49
3	7,800	29	14	10	60
4	8,000	40	18	16	55
5	7,200	28	21	18	61
6	6,100	25	13	11	54

For each subject, peripheral blood counts, surface membrane immunoglobulin determination, EC3 rosettes, and sheep erythrocytes rosettes were determined as described in Methods.

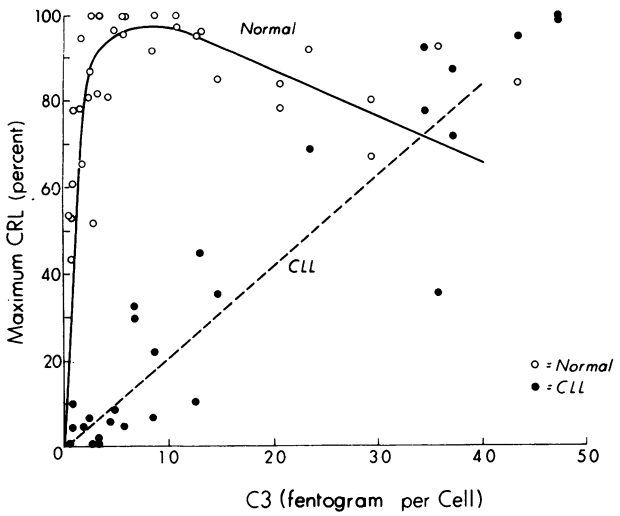

FIGURE 2 The percentage of maximal complement-reactive lymphocytes detected with erythrocytes coated with varying amounts of C3b are shown. This figure represents data from the study of lymphocytes from six normal subjects (O) and five patients with CLL (●). Each point is the average of duplicate membrane-bound C3 measurements and duplicate percent lymphocyte rosette determinations for a given patient.

Though the total lymphocyte number in each assay was kept constant, CLL lymphocytes generally contained a greater fraction of complement reactive cells. Thus, it could be argued that this effect is due to alterations in the ratio of CRL to erythrocytes. To test this possibility the rosette assay was performed at concentrations of erythrocytes varying from 1×10^7/ml to 2.5×10^7/ml, all containing 5 fg of C3/cell. No significant change in the percentage of CLL CRL detectable at this C3 concentration was found. Moreover, two of the CLL patients included in this study had percentages of CRL approximating normal (21 and 23%). Lymphocytes from these two patients (one such study is shown in Fig. 1), likewise exhibited this decreased sensitivity.

To verify that the above binding was mediated by C3b, the effect of the conversion of erythrocyte-bound C3b to C3d upon rosetting was studied. EC with 5 fg of C3, predominantly in the form of C3b, were reincubated in undiluted autologous human serum in the absence of antibody at 37°C for 2 h. This procedure has been shown to convert erythrocyte-bound C3 to C3d (14). Inasmuch as antibody is no longer present, no additional complement activation occurs. The ability of these erythrocytes to form rosettes with CLL and normal lymphocytes was restudied. As shown by a typical experiment in Table II, this incubation essentially eliminated all binding of the EC3 to normal lymphocytes. This confirms that at low concentrations of C3 on erythrocytes, binding by normal lymphocytes is predominantly via C3b. The binding of either of these types of EC3 by CLL lymphocytes was small.

DISCUSSION

Evidence that lymphocytic complement receptors play some role in immune recognition has revealed along several lines. Cobra venom-decomplemented animals failed to produce antibodies to some antigens when injected during the hypocomplementemic period (9). Thus, Pepys postulated that complement activation is necessary for humoral recognition of some antigens (9). It has been further suggested that lymphocyte activation proceeds by two sequential steps, antigen receptor binding serving as the first signal, and C3 receptor activation being the second (27). Recent studies from this laboratory have shown that the interaction of complement-coated red cells, in the absence of foreign antigens, causes stimulation of RNA and protein synthesis in normal CR+ lymphocytes (28). On the other hand, Moller and Coutinho have suggested that B lymphocyte-C3-receptors may serve a function of antigen focusing within lymph nodes (8). We find here that receptors on normal human lymphocytes are able to bind to cells coated with extremely small quantities of C3, suggesting a marked sensitivity for interaction with complement-coated antigens.

Patients with CLL accumulate a large number of lymphocytes which share some surface characteristics with normal B lymphocytes. These cells possess surface-bound immunoglobulins and do not form rosettes with unsensitized sheep cells (29, 30). Initial conclusions by different investigators concerning the reaction of these CLL lymphocytes with complement were in disagreement. Some researchers felt that CLL lymphocytes did not react with complement-coated cells while others found reactivity (31–33). Subsequently, it has been established that a large proportion of CLL lymphocytes, varying from patient to patient, do react with complement-coated erythrocytes, but that this re-

TABLE II
Lymphocyte Rosetting with EC3b and EC3d

Coated erythrocytes	Lymphocytes forming rosettes	
	Normal	CLL
	%	
Before reincubation in serum (EC3b)	10.5	3
After reincubation in serum (EC3d)	0	1

EC3b coated with 5.3 fg C3 per cell were prepared as described in text. These cells were reincubated in fresh human serum to convert the C3b to C3d. Rosettes were then formed using EC of each type with lymphocytes from a normal subject and a CLL patient.

activity depends upon the source of complement used to coat the indicator erythrocytes (34). Ross et al. found that CLL lymphocytes reacted poorly with human complement in comparison to mouse complement (34). This was in contrast to the reactivity of normal complement reactive lymphocytes; thus, Ross et al. postulated that altered complement receptors are present on the CLL cells (34). We have measured binding of human complement to CLL lymphocytes. In patients with CLL, the complement reactive cells, although sometimes present in greater numbers than in normal subjects, require a far greater quantity of C3 coating the indicator erythrocytes to achieve maximal rosetting. This result suggests a decreased affinity of the C3 receptors on CLL cells for C3. The difference did not seem to be due to the generally larger percentage of CRL in the CLL patients, because varying the ratio of EC to lymphocytes did not alter the proportion of CLL complement reactive cells detected with lower levels of C3. Also, the fraction of CRL present in two CLL patients was close to the range of normal, but the difference in sensitivity to lightly complement-coated erythrocytes remained.

Dameshek described CLL as a disease of the accumulation of immuno-incompetent lymphocytes (35). As such, it represents an acquired immunodeficiency disease. As this disease progresses, the patients develop increased susceptibility to infection, decreased serum immunoglobulins, and increased autoantibodies (36). These characteristics are similar to those of other immunodeficiency diseases. A number of abnormalities of the surface characteristics of leukemia lymphocytes have been described, including abnormal lectin binding (37) and altered intramembraneous Ig redistribution ("capping") (24). Here we measure another manifestations of such altered surface function specifically involving complement binding. Although we cannot be certain whether the abnormality in complement receptors described here represents a cause of immuno-incompetence, or merely a nonfunctional change in malignant cell line, the failure of CLL lymphocytes to bind normally to substances coated with C3 may contribute to the immunodeficiency exhibited by such patients. Moreover, we cannot determine whether the remaining normal complement reactive lymphocytes, if any, in such patients, have altered binding properties.

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