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REACTIVITY OF RED CELL ELUATES AND SERUMS IN PATIENTS WITH ACQUIRED HEMOLYTIC ANEMIA AND CHRONIC LYMPHOCYTIC LEUKEMIA *

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The purpose of this study was to define further the immunologic characteristics of the Coombs-positive red cell eluates and serums of patients with acquired hemolytic anemia and chronic lymphocytic leukemia by determining whether they are reactive with and related to autologous neoplastic lymphocytes. These erythrocyte-coating globulins have provoked a great deal of investigative and clinical interest and as a result certain of their properties are well recognized. It is generally agreed that they are heterogeneous globulins (1) which sensitize human erythrocytes both *in vitro* and *in vivo* without regard to cell type (2) and that they may stimulate antiglobulins when injected into an appropriate recipient (3). Nevertheless, the immunologic origin of these proteins has not been adequately delineated.

The frequency with which Coombs-positive acquired hemolytic anemia occurs in chronic lymphocytic leukemia as opposed to other leukemias and lymphomas (4–7) and the improvement which may follow the administration of lympholytic drugs, such as the adrenocortical steroids (8), suggested that the autologous, circulating, neoplastic lymphocyte might function as an antigenic challenge in these patients. This concept has been discussed on prior occasions (9, 10), but experimental evidence to sustain this supposition has been inconclusive.

The technique of immune adherence (11), in which an antigen either macroscopically or microscopically adheres to a primate red cell or non-primate platelet indicator, only in the presence of complement and specific antibody, was employed in this investigation because its suitability for the detection of antigen-antibody reactions has been well demonstrated (12–14).

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METHODS AND MATERIALS

A. Subjects

1. Patients with chronic lymphocytic leukemia. A total of 9 male patients, varying in age from 56 to 72 years, was employed in this study. Despite the fact that the peripheral blood lymphocyte morphology was variable and not all patients exhibited the small, characteristic, dark-staining lymphocyte as the predominant cell type at all times, a diagnosis of chronic lymphocytic leukemia was made in all patients because of their age and prolonged clinical course. Except for the patient who died as the result of an acute blastic crisis 9 months after diagnosis of his blood disorder, the patients were known to have had leukemia for a minimum of 12 months. Two patients were, for all intents and purposes, clinically well and had as significant abnormalities only an elevated total leukocyte count with an absolute lymphocytosis, and minimal or no splenomegaly or lymphadenopathy. There was marked splenomegaly and peripheral lymphadenopathy in four patients with lymphoblasts, immature lymphoid elements resembling reticular lymphocytes, and prolymphocytes in their peripheral blood. In two patients there was moderate lymphadenopathy and splenomegaly, moderate anemia, and mature-type "cleft" leukemic lymphocytes in the peripheral blood. The presence of marked splenomegaly and nucleolated lymphoid cells in the peripheral blood of one patient without lymphadenopathy suggested that his disease began as a primary splenic lymphocytic lymphoma with eventual discharge of these neoplastic lymphocytes into the peripheral blood. Six patients did not receive antileukemic therapy during the study, but serum and red cell eluate reactivity were evaluated in one patient during prednisone therapy, in one patient before and after the administration of prednisone and chlorambucil, and in one patient before and after he was treated with chlorambucil alone.

2. Controls. This group consisted of 8 subjects: 2 healthy laboratory technicians; and 6 patients, one each with sickle cell trait, diabetes mellitus adequately controlled, chronic glomerulonephritis with azotemia, rheumatoid arthritis with mild anemia, post-hepatitic cirrhosis, and peptic ulcer.

B. Tests for erythrocyte sensitization

Qualitative direct and indirect Coombs test (15) were performed on leukemic and control patients with a po-

tent, broad-spectrum antihuman globulin serum.[1] This test was performed in duplicate, both with and without fresh human serum as a complement source, because of prior observations which suggested that complement increases the sensitivity of the Coombs reaction (16, 17). Human serum was employed, as opposed to guinea pig serum, in a deliberate attempt to avoid the introduction of a heterologous agent that might interfere with the evaluation of the test.

Quantitative direct Coombs tests were determined by the ability of serially diluted red cell eluates to resensitize normal erythrocytes. The technique consisted of incubating 0.1 ml of a 2 per cent suspension of normal, type O, Rh negative erythrocytes, 0.3-ml aliquots of the red cell eluate (obtained as outlined below) serially diluted with normal saline, 0.25 ml of human complement diluted 1:10 with gelatin-veronal buffer (GVB), and 0.25 ml GVB in a 37° C water bath for 1 hour. The mixture was then centrifuged and washed with saline four times, the supernate removed, and the Coombs test performed.

Quantitative indirect Coombs tests were similarly performed by incubating 0.25 ml of a 2 per cent suspension of type O, Rh negative erythrocytes, 0.75-ml aliquots of patient serum serially diluted with normal saline, and 0.25 ml of fresh human complement diluted 1:10 with GVB. After incubation in a 37° C water bath for 1 hour, the reactants were centrifuged, washed, and the Coombs test performed. Serum and eluate dilutions varied from full strength to 1:72 and were used in these concentrations because they afforded optimum reactivity in the test. The Coombs antiglobulin serum was used undiluted, at full strength, in all determinations. In all instances the Coombs reaction was considered positive when either macroscopic or microscopic red cell agglutination was observed.

C. Preparation of reactants for immune adherence (IA) assay

1. Complement. Normal, human, type AB, Rh negative serum was used as complement source. It was kept at −70° C in 1-ml aliquot portions in screw-top vials. After quick thawing it was used in a constant dilution of 1:40 with GVB.

2. Lymphocytes. To prepare leukemic lymphocytes, 10 ml of blood was aspirated into a syringe containing 0.5 mg heparin, expressed into a clean, dry 15 × 150 mm screw-top test tube, and centrifuged at room temperature in a PR-2 International centrifuge (head no. 269) at 2,000 rpm (900 G) for 20 minutes. The supernatant plasma and ¾ of the red cells deposited in the lower portion of the tube were transferred to a similar test tube with a capillary pipet. The mixture was agitated gently to insure adequate mixing and incubated in a 37° C water bath for 45 minutes. The supernatant plasma was centrifuged at room temperature at 600 rpm (80 G) for 7 minutes. The supernatant plasma was discarded and the resultant leukocyte mixture resuspended in 5 ml of cold

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[1] Ortho Pharmaceutical Corp., Raritan, N. J.

physiologic saline. This suspension was permitted to precipitate at room temperature for approximately 30 minutes or until the major portion of the leukocytes settled out. The supernatent was then discarded and the leukocyte sediment resuspended in 5 to 10 ml of normal saline.

In order to obtain a lymphocyte suspension as free as possible of contaminating polymorphonuclear leukocytes, a modification of the glass-wool column leukocyte separation technique (18) was employed. This method removed approximately only 50 per cent of the contaminating polymorphonuclear leukocytes but is, nevertheless, one of the more practical methods available for this type of separation. The lymphocyte suspension, after filtration through the glass-wool column, was washed three times with GVB. These procedures did not alter the morphologic integrity of the lymphocytes. A 2 per cent lymphocyte suspension (0.2 ml packed lymphocytes suspended in 9.8 ml GVB) was considered to be full strength, and serial dilutions up to 1:16 were made with GVB to be used in the immune adherence assay.

To obtain lymphocytes from the control patients, 40 ml of heparinized blood was aspirated, divided into 10-ml aliquots, and treated in a manner similar to that for leukemic lymphocytes. The combined lymphocyte yields of 4 tubes were pooled and, because technically it was not possible to obtain as many lymphocytes from control as from leukemic patients, the lymphocyte suspension was adjusted to 20 per cent transmittance on a Beckman DU spectrophotometer at a wave length of 400 mμ. This served as the full-strength concentration in the control assays, and subsequent dilutions up to 1:16 were made with GVB.

3. Materials to be used as antibody. In order to evaluate the lymphocyte-antigen hypothesis, heterologous lymphocyte antibody was made by injecting human leukemic lymphocytes into rabbits. A 3-ml suspension of lymphocytes, containing a total of 7.5 × 10⁶ cells, was injected twice weekly for 3 weeks into the marginal ear vein of albino rabbits weighing approximately 2 kg. The animals were rested for 7 days; 10 ml of blood was then harvested from the ear vein. The blood was allowed to clot and the serum was removed and stored in 2-ml aliquots at −35° C. Prior to use in the assay the serum, containing the heterologous neoplastic antibody, was heated to 56° C for 30 minutes to inactivate complement. It was then subsequently absorbed, in succession, with erythrocytes from the lymphocyte donor and with the IA indicator erythrocytes (2 parts red cells to 1 part serum) at 0° C for 45 minutes to remove any heterologous erythrocyte antibody. The absorption was repeated until full-strength rabbit antiserum no longer caused microscopic red cell agglutination. Heterologous lymphocyte antibody in immune adherence was used in dilutions from full strength to 1:2,560, made with GVB.

Red cell eluates were prepared by acid elution (2) after washing the erythrocytes six times with normal saline at room temperature. Reactivity of the washings was not evaluated by conventional Coombs or IA tests. Eluate reactivity from unwashed cells or from red cells

washed fewer than six times was not determined. This method, after the final step in the procedure, resulted in approximately 20 to 30 ml of a brownish-tinged fluid which was dialyzed overnight in cellophane casing (½ inch diameter)[2] against a 15 per cent solution of Plasdone-C (polyvinylpyrrolidone).[3] The residuum, amounting to about 0.5 ml of concentrated eluate, was diluted to a standard volume of 3 ml with GVB and was considered to be "full-strength eluate." Eluates in immune adherence were used in dilutions from full strength to 1:16,384, made with GVB.

Serums to be used as antibody were prepared as follows: 15 ml of blood was placed in a 15 × 150 mm dry, clean test tube and allowed to clot at room temperature for 1 hour; the clotted blood was refrigerated at 4° C for 4 hours. The blood was then centrifuged at room temperature and the serum removed with a capillary pipet and stored in 2-ml aliquots at − 35° C until used. Serum dilutions in immune adherence ranged from full strength to 1:640, made with GVB. All antibody dilutions were devised to give optimum reactivity in the assays.

4. Indicators. Indicator red cells were collected, stored, and employed as previously described in detail (14). The final constant red cell concentration was adjusted to an optical density of 0.390 at a wave length of 541 mμ with a slit opening of 0.02 on a Beckman DU spectrophotometer. Guinea pig platelets were collected and prepared as previously described (19). They were standardized to 20 per cent transmittance at a wave length of 400 mμ on a Beckman DU spectrophotometer and used in this constant concentration.

D. Assay

Heterologous antibody reactivity was determined in Kontes hemagglutination tubes as previously described (14). In a checkerboard titration pattern, 0.25-ml aliquots of serially diluted heterologous lymphocyte antibody were added slowly to 0.25-ml portions of serially diluted leukemic lymphocytes. Complement, 0.5 ml, diluted 1:40 with GVB, was added to each tube and, after

[2] Arthur H. Thomas Co., Philadelphia, Pa.
[3] Antra Chemical Div. General Aniline and Film Corp., Charlotte, N. C.

a 15-minute incubation period in a 37° C water bath, 0.1 ml of the standard red cell indicator suspension was added to each tube with gentle mixing. After another 45-minute incubation, IA patterns (0 to 4+) were determined macroscopically and confirmed by wet coverslip preparations under dark-field microscopy. An example of this titration is illustrated in Table I.

Red cell eluate and serum reactivity were determined as previously described in detail (19). With separate checkerboard titrations for each antigen, serial dilutions of red cell eluates and serums were added in 0.25-ml aliquots to 0.25-ml portions of serially diluted erythrocyte and lymphocyte antigen in 2-ml screw-top vials. The vials were rotated at 6 rpm in a 37° C incubator for 10 minutes. They were removed from the incubator, 0.5 ml complement (1:40) was added to each vial, and then were reincubated on the rotator. Then 0.1 ml of platelet indicator suspension was added to each vial and the incubation repeated for 40 minutes. Wet coverslip preparations were examined microscopically for immune adherence. The reaction was read as negative when no antigen adhered to the platelets, as a trace when less than 10 per cent adhered, + 1 for IA of 10 to 25 per cent, + 2 for IA of 25 to 50 per cent, + 3 for IA of 50 to 75 per cent, and + 4 with IA of more than 75 per cent of the antigen.

E. Tests for lymphocyte sensitization

During the course of the study, when initial results suggested that the Coombs-positive globulins might be related to neoplastic lymphocytes, qualitative Coombs tests, as described previously, were performed on both leukemic and control lymphocytes.

RESULTS

A. Tests for erythrocyte sensitization. Of the nine patients with leukemia, five had a positive direct Coombs test, two had a positive indirect test, one was positive both direct and indirect, and one patient was completely negative. In all instances fresh serum either increased the positivity of a reaction or converted a negative test to a positive

TABLE I

Immune adherence assay of heterologous lymphocyte antibody

Antigen*		Antibody dilution†										
Conc.	Dilution	Undil.	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560
% 2	Undiluted	+4	+4	+4	+4	+4	+3	+3	+3	+2	+2	+2
2	1:2	+3	+3	+4	+4	+3	+3	+2	+2	+2	+2	+2
2	1:4	0	+1	+2	+3	+3	+2	+2	+2	+1	+1	+1
2	1:8	0	0	+1	+1	+2	+2	+2	+1	+1	0	0
2	1:16	0	0	0	+1	+1	+1	+1	+1	0	0	0

* Human leukemic lymphocytes.
† Heterologous rabbit antiserum.

Qualitative and quantitative Coombs
tests in patients with leukemia

Patient	Qualitative Coombs test		Quantitative Coombs test	
	Direct*	Indirect	Direct†	Indirect‡
1	0	0		
2	dt +	0	1:4	
3	bt +	0	1:32	
	at +	+	1:4	1:4
4	bt 0	+		1:8
	at +	+	1:16	1:4
5	+	0	1:48	
6	+	0	Undil. only	
7	+	0	1:4	
8	0	+		1:24
9	+	0	1:2	

* During treatment, dt; before treatment, bt; after treatment, at.
† Dilution of red cell eluate reacting with undiluted Coombs antiserum after resensitization of normal, type O Rh negative erythrocytes.
‡ Dilution of serum reacting with undiluted Coombs antiserum after resensitization of normal, type O, Rh negative erythrocytes.

result and therefore only this test is recorded. Quantitative Coombs tests performed with red cell eluates and serums varied in titer from full strength to a 1:48 dilution. These results are summarized in Table II.

Of the eight control subjects six had negative direct and indirect Coombs reactions. However, the patients with peptic ulcer and post-hepatitic
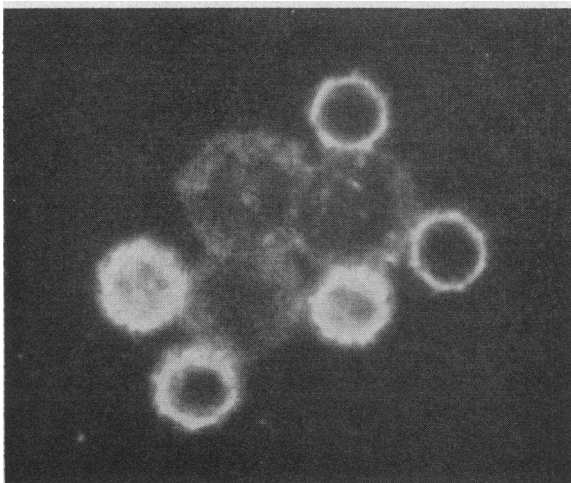

FIG. 1. UNDER DARK-FIELD MICROSCOPY, THE LEUKEMIC LYMPHOCYTES IN THE CENTER OF THE FIELD ARE ADHERENT TO THE MORE LUMINESCENT, PERIPHERAL INDICATOR RED CELLS (×450).

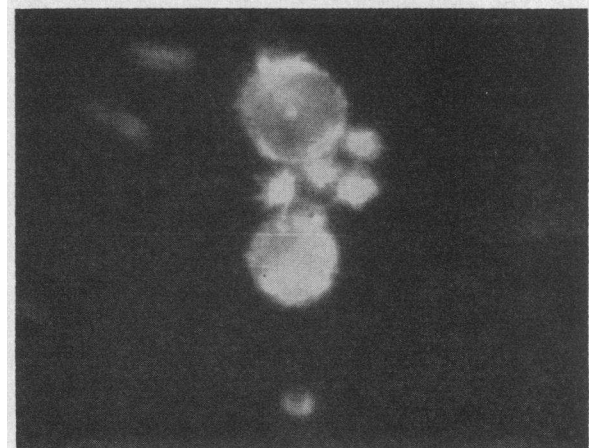
FIG. 2. IMMUNE ADHERENCE OF THE AUTOLOGOUS, LEUKEMIC LYMPHOCYTES TO THE STELLATE, INDICATOR GUINEA PIG PLATELETS UNDER DARK FIELD (×450).

cirrhosis both had positive direct Coombs tests. Neither patient, however, was anemic and neither patient had an elevation of the reticulocyte count. The red cell eluate from the patient with cirrhosis resensitized normal erythrocytes only when undiluted, and the patient with post-hepatitic cirrhosis had a resensitization titer of 1:8.

B. Immune adherence assays. Heterologous leukemic lymphocyte antibody was reactive (+ 2) in a serum dilution up to 1:2,560 and indicates that IA is a valid method for detecting lymphocyte antibody (Figure 1). The + 2 reaction in this dilution is evidence that the antibody most probably would have retained its reactivity for at least another tube dilution.

TABLE III

Leukemic red cell eluate and serum reactivity
in immune adherence (IA)

IA re-activity*	Coombs-positive eluates†	Coombs-positive serums†
	no.	no.
1:4	1	0
1:8	1	0
1:32	1	0
1:48	1	0
1:72	1	1
1:128	1	0
1:192	0	2
1:384	0	1
1:16,384	2	0
Total	8	4

* Dilution of eluate or serum reactive in IA with autologous leukemic lymphocytes.
† Represents 2 patients studied before and after antileukemic therapy.

TABLE IV

Clinical variables and immune adherence reactivity

Patient	Therapy	Predominating cell type *	Anemia	IA reactivity †	
				Eluate	Serum
1	0	MTL	0	0	0
2‡	Prednisone	MTL	0	1:72	0
3§	Chlorambucil and prednisone	bt: lymphoblasts, prolymphocytes	Severe	1:16,384	0
		at: ML, occ. prolymphocyte	Mild	1:8	1:192
4	Chlorambucil	bt: nucleolated retic. lymphocytes	Severe	0	1:192
5	0	at: no change	Severe	1:48	1:72
5	0	ML, prolymphocytes, occ. lymphoblast	Severe: patient died	1:16,384	0
6	0	MTL	0	1:4	0
7	0	MTL	Severe: patient died	1:128	0
8	0	MTL	Moderate	0	1:384
9	0	MTL	Mild	1:32	0

* MTL, mature-type lymphocyte; ML, mature lymphocytes.
† Dilution of eluate or serum reactive with autologous, neoplastic lymphocytes.
‡ This patient was admitted with icterus, severe anemia, and septicemia. He showed an excellent therapeutic response. It is felt that the titer of 1:72, obtained after his discharge, would have been much higher if it had been opportune to study him during the acute phase of his illness.
§ This patient, subsequent to completion of this investigation, developed a fulminating lymphoblastic leukemia and expired.

Immune adherence was present when Coombs-positive red cell eluates and serums were reacted with autologous, leukemic lymphocytes, indicating that an antigen-antibody reaction had taken place (Figure 2). With both the eluates and serums the highest dilution giving a + 1 reaction is reported as the end point of the assay. Reactivity of eluates varied from 1:8 to 1:16,384 and that of serums from 1:72 to 1:384. These findings are summarized in Table III. Eluate and serum reactivity were influenced by the clinical variables of morphologic maturity of the lymphocyte, the severity of the leukemic process, and whether the patient had received antileukemic therapy. In general, but not without exception, antibody reactivity was more marked when the lymphocytes were immature and when the clinical state of the patient was poor. Reactivity appeared to diminish during or after treatment. However, in Patient 3 a marked drop in eluate reactivity was accompanied by a moderate rise in serum reactivity, and in Patient 4 the reverse occurred, although this change was not so pronounced. These observations are summarized in Table IV. In both instances the alterations were reflected in similar changes in the direct and indirect Coombs reactions (Table II). In addition, two Coombs-positive red cell eluates and serums (Patients 3 and 4) were reactive in a 1:2 dilution in IA with non-leukemic, control lymphocytes. Reactivity with these cells was less than that obtained with autologous, neoplastic lymphocytes.

Immune adherence was not observed, indicating the absence of an antigen-antibody reaction, when Coombs-positive red cell eluates and serums were reacted with autologous erythrocytes, when Coombs-negative eluates and serums were reacted with autologous erythrocytes and lymphocytes and, particularly, when nonleukemic, control, Coombs-positive red cell eluates were reacted with autologous lymphocytes. The results of the significant qualitative eluate and serum IA assays are summarized in Table V.

C. Tests for lymphocyte sensitization. Lymphocytes from leukemic patients with positive Coombs

TABLE V

Qualitative immune adherence reactivity *

Antigen	Antibody	Indicator	IA
Human leukemic lymphocyte	Heterol. (rabbit) lymphocyte antiserum	Human red cells	+
Autologous leukemic lymphocyte	CP red cell eluate	GPP	+
Autologous leukemic lymphocyte	CP serum	GPP	+
Autologous erythrocyte	CP red cell eluate	GPP	0
Autologous erythrocyte	CP serum	GPP	0
Autologous nonleuk. lymphocyte	CP nonleuk. red cell eluate	GPP	0

* CP, Coombs-positive; GPP, guinea pig platelets.

tests were agglutinated by the Coombs antiserum in a manner similar to that for erythrocytes. All control lymphocytes gave a negative reaction with the antihuman globulin.

DISCUSSION

The most significant observation of this study was the occurrence of immune adherence when Coombs-positive leukemic red cell eluates and serums were reacted with autologous, neoplastic lymphocytes. This is evidence of an antigen-antibody reaction and suggests an immunologic relationship between these substances as demonstrated by the IA technique.

In view of the fact that the lymphocyte is a complex mosaic of proteins, lipids, and carbohydrates and because various agents such as cold, heat, X-ray, and microorganisms have been postulated as irritants for conversion of normal tissue constituents into antigens (20), it is conceivable that this cell, after being taken up by the reticuloendothelial system, might function as an antigen and provoke an autologous immunologic response in a previously unresponsive organism. In this way the abnormal lymphocyte may be responsible, in part, for the stimulation of these abnormal globulins. It is of particular importance, therefore, that immune adherence did not occur when Coombs-positive red cell eluates from the control patients with peptic ulcer and cirrhosis were reacted with their own lymphocytes. It was of related interest, however, that two Coombs-positive red cell eluates and serums were reactive with control lymphocytes. These latter two observations suggest that nonleukemic lymphocytes are not autoantigenic under non-neoplastic conditions but that normal and leukemic lymphocytes may contain cross-reacting antigens.

Although an immunologic relationship has been demonstrated by the immune adherence method between these Coombs-positive globulins and the intact neoplastic lymphocyte, it is not apparent whether these globulins are directed against the whole autologous lymphocyte, per se, or against its individual integral components. The possibility exists, therefore, that these globulins are wholly or in part stimulated by an externally introduced antigen that is retained and transported by the leukemic lymphocyte which then becomes a conjugated antigen (21). The process of conjugation, during which the original antigen or structure undergoes some alteration, creates new categories of antigen specificity to which patients with leukemia may so respond as to produce abnormal antibody globulins. For example, a virus (22) or virus-like agent (23), containing or composed of foreign protein, may combine with the autologous, leukemic lymphocyte and contribute to or accelerate its malignant transformation. As a parallel example, it has been suggested that the autoantibodies and autoimmunity of ulcerative colitis, demonstrated as tissue precipitins, may result from antigenic stimuli provided by the great number of microorganisms known to invade the tissues in this disease (24).

An alternative hypothesis may be that the red cell, which itself may be altered in leukemia, antigenically stimulates these abnormal lymphocytes to produce red cell antibodies. There are several observations that do not support such an approach, however, but do not rule it out completely. First, it postulates that the lymphocyte synthesizes antibody. While this may be true, in part, there is no complete agreement at present as to which cells are responsible for this function (25). The plasma cell (26), reticuloendothelial cell (27), and mononuclear cell of lymphoid origin (28) have all been implicated in this activity. Furthermore, it presupposes that these cells are immunologically competent, a thesis not in accord with the increased homograft tolerance of patients with lymphoma and related disorders (29), their diminished *de novo* antibody synthesis (30), and their cutaneous anergy (31).

The observation that Coombs-positive red cell eluates did not react with autologous erythrocytes indicates the failure of an antigen-antibody union, as determined by the IA technique, and suggests the absence of an immunologic relationship between the red cell and the globulin. This, of course, still does not rule out erythrocyte antibodies or antibody-like substances which escape detection by this particular serologic assay. However, it should be pointed out that lack of reactivity of the autologous Coombs-positive erythrocyte is not necessarily due to the fact that its surface is globulin coated, because similar red cells have been employed successfully as indicators in consistently reproducible starch antigen-antibody IA titrations (32).

The variability of the antibody reactivity as noted in Table IV is consistent with the observation that, during the evolution of malignant cells, associated histochemical differences may effect cellular antigenicity. Furthermore, the breadth of leukoagglutinin activity stimulated by leukocytes obtained from a single donor (33) favors the concept that leukocytes have a complex antigenic structure. In addition, the patient with the mature-type leukemic lymphocytes had a negative Coombs test and his eluate and serum were not reactive in immune adherence, suggesting that the potential autoantigenicity of the leukemic lymphocyte is partially dependent on its increasing immaturity. In Patient 4, whose disease may have originated as a splenic lymphocytic lymphoma, the disparity between antibody reactivity and the pronounced morphologic cellular immaturity in the peripheral blood may be associated with the delayed discharge of neoplastic cells into the peripheral blood.

The Coombs-positive eluates and serums were more reactive in patients with severe, progressive, and terminal disease than in less seriously ill patients. This implies that during the uncontrolled or resistant phase of leukemia more of these abnormal proteins may be produced, perhaps because the immune mechanism of such patients is capable of recognizing, and therefore regarding as foreign, a degree of change in an autologous cell that is ignored by the immune mechanism of healthy individuals.

Diminution of eluate and serum reactivity was noted in patients treated with adrenocortical steroids and chlorambucil. The precise method by which the adrenocortical steroids ameliorate Coombs-positive acquired hemolytic anemia is not known, but several explanations may be attempted. The steroid hormones perhaps exert their influence by accelerating the rate of removal of lymphoid cells or by inhibiting their production (34) and therefore decreasing the amount of antigenic material to which the immune mechanism is exposed. Another factor may be that the adrenocortical steroids influence the antibody-producing mechanism itself and prevent its responsiveness to antigenic stimuli (35). Finally, the efficacy of steroids may depend on augmenting effective bone marrow erythropoiesis (36). The reactivity-reducing mechanism of chlorambucil, a polyfunc-

tional alkylating compound, may be intimately related to its ability to denature or inactivate cellular nucleoprotein (37). The drug may reduce the total amount of potentially antigenic material or alter the biologic properties of the neoplastic cell so that its antigenicity is decreased.

There are four other observations regarding these globulins which require comment. Patient 4, prior to therapy, and Patient 8 had only indirect positive Coombs tests. It may be suggested that this is unusual and represents an acquired isohemagglutinin as the result of blood transfusions. However, it is more likely that this is a type of cold antibody, often found in reticuloendothelial malignancies, which did not bind to the red cell at body temperature or is a globulin similar to the macromolecular cold antibody (38). The fact that it resensitized type O, Rh negative erythrocytes makes it less probable that this is a type-specific antibody stimulated by isosensitization. The associated change in the location of the globulins in Patients 3 and 4 after therapy indicates not only that their reactivity was modified but also suggests that the red cell membrane or the structures upon which globulin binding is dependent may have been altered by the adrenocortical steroids and the chlorambucil. The agglutination of leukemic lymphocytes from patients with Coombs-positive acquired hemolytic anemia by the Coombs antiserum suggests that these cells, as well as erythrocytes, are coated with abnormal proteins. Finally, the observation of the marked reactivity of two of the eluates (dilution to 1 : 16,384) does not necessarily imply that this is characteristic of the eluates, per se, or that the eluate globulins differ in potency from the serum globulins which artificially resensitize erythrocytes *in vitro*. It more likely suggests that for reasons as yet undisclosed, under certain circumstances, more globulin coats the erythrocyte than remains floating free in the serum. This is supported by instances, as noted in Table IV, in which the serum reactivity was obviously higher than that of the eluates.

These observations do not completely explain the genesis of the Coombs-positive globulins in this type of acquired hemolytic anemia. It is possible that the globulins will cross react with antigens that are as yet unknown, aside from the intact leukemic lymphocyte. In addition, they may

react more strongly with some fractions of the lymphocyte and be weakly or nonreactive with others. These experiments, however, represent another approach to an unsolved problem and may help to explain the immunologic alterations in this syndrome.

SUMMARY

1. The most important observation of this study was that the Coombs-positive red cell eluates and serums of patients with acquired hemolytic anemia and chronic lymphocytic leukemia reacted in immune adherence with autologous leukemic lymphocytes. This is evidence of an antigen-antibody reaction and suggests an immunologic relationship between these substances, as demonstrated by the immune adherence method.

2. On the other hand, red cell eluates and serums did not react with autologous erythrocytes nor did control, nonleukemic red cell eluates react with control, nonleukemic lymphocytes, suggesting the absence of a similar relationship between these elements.

3. Mild reactivity of two Coombs-positive red cell eluates and serums with control lymphocytes suggests that normal and leukemic lymphocytes may contain cross-reacting antigens.

4. It is suggested that the autologous leukemic lymphocyte may be one of the factors that stimulate the development of the Coombs-positive globulins.

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