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Clinical importance of circulating immune complexes in human acute lymphoblastic leukemia

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Summary. A total of 122 sera from acute lymphoblastic leukemia (ALL) patients were analyzed for circulating immune complexes (CIC) by two methods: the ^{125}I-C_1q binding assay and the polyethylene glycol precipitation test (PEG). The results were correlated with induction, remission and relapse stages of the disease. Using the first method the levels of CIC in induction were 15.18 ± 9.15, with 19/29 positive cases (65.50%), $P < 0.001$ compared with controls. In the remission phase the levels were 9.02 ± 5.62, 11/45 (24.49%) nonsignificant P value, and in relapse they were 16.14 ± 11.17 28/48 (58.33%) $P < 0.001$. The PEG precipitation test results were: 0.33 ± 0.10, 8/22 (36.36%); 0.24 ± 0.11, 10/48 (20.83%) and 0.28 ± 0.10, 6/28 (21.42%), respectively. Thus the values of CIC as measured by PEG in the three clinical of phases ALL did not differ significantly from controls. This contrasts with results obtained by the radioiodinated C_1q binding assay, where the incidence of positive values was significantly higher in induction and in relapse and lower in the remission phase. These observations were extended in sequential vertical studies performed in a group of patients. These results suggest that raised CIC detected by the ^{125}I-C_1q method may reflect a progressive state in ALL and that quantitation of these immune complexes may provide an adequate biochemical marker for prognosis.

Introduction

During the last 10 years increasing interest has developed in research studies concerning the possible association of immune complexes with malignant diseases. It has been observed that the inhibition of cell-mediated immune reactions seems to be related to several malignant tumors. There is evidence showing that some soluble factors are inducers of this cellular immune inhibition [18]. These immune complexes may be formed by tumor-associated antigens and specific antitumor antibodies [13]. Their suppressive action on cell-mediated immunity has not been elucidated, although they do appear to block effector T cell functions [20].

There are several reports concerning the correlation of circulating immune complex (CIC) levels and the clinical status of malignant disease, including leukemia [1, 5, 8, 15, 17]. It has been suggested that immune complexes may induce tumor progression changing the immune response of the host against malignant cells [2]. The present investigation was undertaken to determine more clearly the clinical significance of CIC in acute lymphoblastic leukemia (ALL). The patients were studied over a 3-year period to try to discover a correlation between CIC levels and the clinical stage of the disease.

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Materials and methods

Patients. A total of 122 serum samples from 55 children with ALL were studied for the presence of immune complexes using two methods: the ^{125}I-C_1q binding assay [29] and the polyethylene glycol precipitation test (PEG) [10]. Sera from 29 patients were analyzed at the time of diagnosis, 45 during remission and 48 at relapse. Also a sequential study of CIC was performed in a group of patients over a period of several months.

Patients were treated according to uniform therapeutic regimens (GATLA: Grupo Argentino para el Tratamiento de la Leucemia Aguda). Patients were classified as being in induction phase, complete remission or relapse. At the time when blood samples were taken, most of the patients were being treated as follows. Briefly, treatment for induction of remission consisted of daily courses with prednisone, L-aspariginase over 4 weeks; vincristine and daunorubicin once per week with a total of four doses followed by cyclophosphamide plus 4 weeks receiving cytarabine and 6-mercaptopurine. Central nervous system involvement was prevented by intrathecal injection of methotrexate and dexamethasone. This therapeutic protocol also included a re-induction of remission treatment. It consisted of continuous administration of dexamethasone plus vincristine and adriamicin weekly and L-aspariginase on each of 4 days over 4 weeks; it was followed by a continuous 2-week treatment with cyclophosphamide and daunorubicin; it was completed with intrathecal methotrexate and dexamethasone.

Serum samples. Blood was taken aseptically from patients and allowed to clot at room temperature for at least 60 min. It was centrifuged at 1,500 g for 15 min, and sera were then aliquoted into Durham tubes and stored at $-70\,^{\circ}$C until used. Although it has been shown that freezing and thawing do not alter CIC levels [25] each serum sample was thawed out only on the day of the test. As controls, serum samples were collected from 46 normal si-

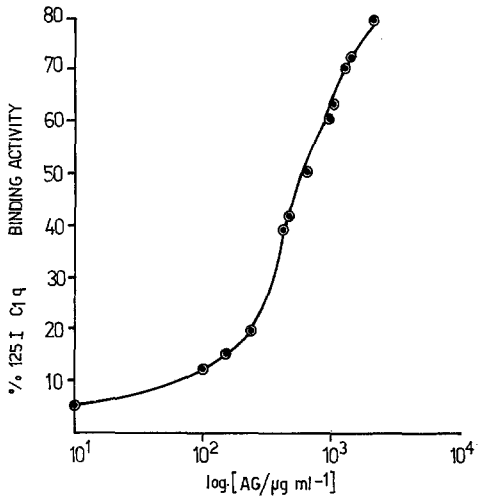

Fig. 1. C_1q binding activity by human gamma globulin aggregates suspended in normal human serum. CIC normal value of 9.00% corresponded to 55 µg/ml of aggregates

blings of leukemic patients or nonmalignant patients of either sex admitted to The Children's Hospital for hernia, phimosis, ectopic testes and idiopathic scoliosis corrective surgery. These controls had no history of infection for a 2-week period prior to blood sampling. All control samples were collected in the same way as in the experimental group.

Detection of circulating immune complexes ^{125}I-C_1q binding assay. This assay for CIC detection is related to the property of immune complexes to react with one of their receptors, the first complement component. This method measures the quantity of radioiodinated C_1q combined to immune complexes in the test serum. The C_1q was isolated from a pool of fresh human serum as previously described [28] and radiolabeled with ^{125}I-C_1q by means of the lactoperoxidase method [14].

The ^{125}I-C_1q; binding assay was performed as previously described [29], together with the slight modification by a short incubation of the serum samples with heparin before the first step. This modification suggested by Baldwin et al. [3] improves the discrimination between patients and control studies. In each test between five and seven normal serum samples were tested to determine the normal range, all sera were tested in duplicate and in three different assays. Also, in each assay as a positive control, heat aggregated human immunoglobulin (63 °C for 20 min) obtained from Cohn fraction II (Sigma, Chemical Company, St. Louis, MO. 63178, USA) was used diluted in normal human serum. Results were expressed as the percentage of ^{125}I-C_1q in experimental precipitates compared to ^{125}I-C_1q in precipitates of tubes containing 50 µl normal human serum and 50 µl ^{125}I-C_1q precipitated with 20% trichloroacetic acid. The normal value of CIC by this assay was 9.00 ± 2.00 ($x \pm 2SD$). This value was equivalent to 55 µg/ml heat aggregated human immunoglobulin (Fig. 1). The ^{125}I-C_1q binding activity was considered elevated if the results were 2SD above the normal range.

The polyethylene glycol precipitation test (PEG). This was a modification of the technique previously described [10, 11, 24]. Briefly, 0.1 ml test sera was diluted to 0.9 ml in borate buffer 0.1 M, pH 8.4 and 1.0 ml of 8% PEG in borate buffer was added. The mixture was incubated at 4 °C for 18 h and then centrifuged at 2,000 g at 4 °C for 30 min. The precipitate was suspended and vortexed in 2 ml PEG. Samples were centrifuged at 2,000 g for 30 min. The supernatant was discarded and the precipitate dissolved in 0.1 ml NaOH by agitation; the optical density was determined at 280 nm. The normal value was 0.22 ± 0.05 ($x \pm 2SD$), and therefore values over 0.27 were considered as positive.

Statistical analysis was carried out using the Student's *t*-test.

Results

CIC levels using the radioiodinated C_1q binding test

In order to determine the extent to which CIC levels could be used to assess prognosis, a sequential study was performed in a group of ALL patients, 29 in induction, 48 in relapse and 45 in remission. The CIC were determined in a total of 122 sera from 55 ALL patients, and the results are shown in Table 1. Under the induction of remission treatment (blastic stage) CIC values were 15.78 ± 9.15 with 19/29 positives (65.50%) ($P < 0.001$ compared with normals). In remission the mean value was $9.02 \pm 5{,}62$, 11/45 positives (24.49%), and in relapse phase the mean value was 16.59 ± 11.17, 28/48 (58.33%) positives ($P \pm 0.001$ in relation to normals). Compared with the sera from 46 controls the mean value of C_1q binding activity was significantly increased in patients in induction and in relapse stages. Conversely, CIC levels were no longer detectable in sera from the remission phase group and similar to controls ($P > 0.5$).

Figure 2 shows the CIC results obtained in patients in relapse followed by a remission phase. In most of them the raised CIC levels were prevalently associated with the acute phase of leukemia with normal values in remission. In Fig. 3 the CIC values obtained in sera from patients in

Table 1. Incidence of circulating immune complexes in patients with ALL according to the stage of disease using the ^{125}I-C_1q bindig assay

Stage	*n*	$\bar{x}$	$^{\delta}n-1$	*P**	+/totals	%
Induction	29	15.18	9.15	<0.001	19/29	65.50
Remission	45	9.02	5.62	N.S.	11/45	24.49
Relapse	48	16.14	11.17	<0.001	28/48	58.33

*p value comparing with controls
Normal value: 9.00 ± 2.00, ie., $\bar{x} + 2SD$; 11% is the upper limit of normal values
N.S.: not significant ($P > 0.5$)

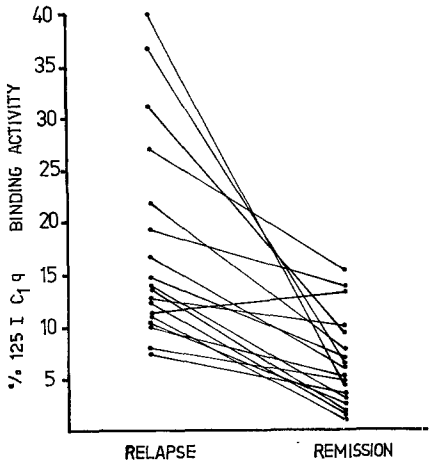

Fig. 2. Evolution of serum C_1q binding activity in 18 patients with ALL according to two stages of disease: relapse and remission. Normal value was 9.00 ± 2.00

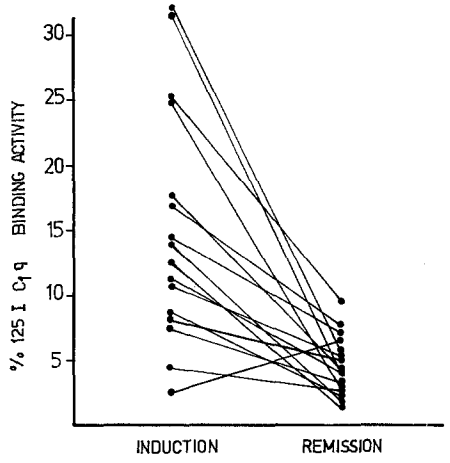

Fig. 3. Evolution of serum C_1q binding activity in 16 patients with ALL according to two stages of disease: induction and remission. Normal value was up to 9.00 ± 2.00

induction followed by remission are shown. Results were significantly lower when patients went into remission.

In a small group of ALL patients a sequential study of CIC was done in the three phases of disease: induction, remission and relapse, and the results are shown in Fig. 4. Most of the patients showed high levels in induction phase. Only 1 presented a low value. In the 6 patients CIC levels were normal in remission while in relapse 4 yielded high values and 2 border line percentages.

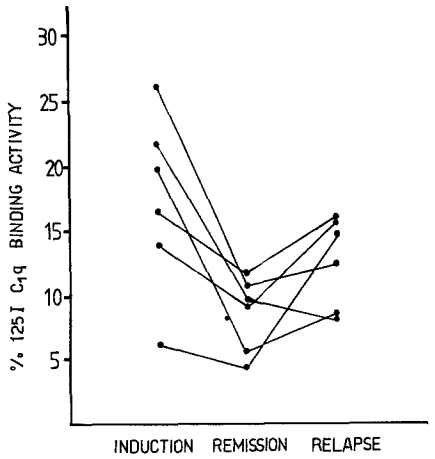

Fig. 4. Evolution of serum ^{125}I-C_1q binding activity in six patients with ALL according to three phases of the disease: induction, remission and relapse

CIC levels using the PEG precipitation test

Most of the sera studied using the radioiodinated C_1q binding test were tested for CIC by the PEG precipitation method. A sequential study was performed in the three phases of disease. Patients in induction showed an optical density of 0.33 ± 0.10 (36% positives), in remission 0.24 ± 0.11, 10/24 (20%) and in relapse 0.28 ± 0.10, 3/14 (21.42%) (Table 2).

Discussion

This study performed using the ^{125}I-C_1q binding assay and the PEG precipitation test confirms previous reports on the presence of CIC in sera from patients with ALL. These complexes were found to be reactive with C_1q and precipitable by PEG. Results obtained by both methods indicate that patients with ALL have high levels of antigen-antibody complexes. The amount detected is related to the status of disease; in particular a high percentage of immune complexes were found in the sera of ALL patients with a high number of blasts (acute phase of disease). It has been demonstrated that leukemic blasts display large amounts of strong immunogenic leukemia-associated antigens [9]. In ALL there is a proliferation of nondifferentiated lymphocytes and the presence of immune complexes in the plasma of these patients suggests that there are enough lymphocytes which remain capable of inducing antibody response. Therefore, immunogens derived from leukemic cells may form immune complexes when they combine with their corresponding antibody. These immune com-

Table 2. Incidence of circulating immune complexes in patients with ALL according to the stage of disease using the PEG precipitation assay

Stage	n	$\bar{x}$	$\delta n-1$	P*	+/totals	%
Induction	22	0.33	0.10	<0.001	8/22	36.36
Remission	48	0.24	0.11	<0.005	10/48	20.83
Relapse	28	0.28	0.10	<0.001	6/28	21.42

*p value comparing with controls
Normal value: 0.22 ± 0.05, i.e., 0.27 was considered the upper limit of normal values

plexes do not induce vasculitis or serum sickness although they may produce immune complex diseases because of their size. Nevertheless, none of these leukemic patients showed clinical or laboratory data suggesting a membranous glomerulonephritis. Possibly, immune complexes in ALL patients may be considered as terminal stage immune complexes [27] which are unable to fix onto cell membrane receptors or to activate complement components. Therefore, they have lost their injuring capacity. Another fact which is difficult to assess is why the destruction of many malignant cells by chemotherapy does not increase CIC levels.

In the whole group of patients there was a striking difference in CIC levels between induction and remission phases and between relapse and remission with $P<0.001$ for both groups. No differences of CIC levels were observed between patients in induction and in relapse phases. In remission CIC values were decreased, yielding normal levels (9.02 ± 5.62). The present findings correlate with those reported by Hubbard et al. [16] who obtained a clear relationship between CIC positive levels and the progressive state of leukemia using the Raji cell immunoassay. Similar results were found by Carpentier et al. [7] in acute myeloid leukemia using the ^{125}I-C_1q binding assay. Also, Euler et al. [12] using the 3% PEG precipitation test found that the quantification of precipitable immune complexes and their components may be valuable as adjuncts in determining disease activity in Hodgkin's disease. Segal-Eiras et al. [25] found high levels of CIC in osteosarcoma patients, a possible correlation with recurrence/metastasis and the IgG nature of the antibody involved. On the contrary, Minden et al. [19] did not find a relationship between the incidence of relapse and the presence or absence of immune complexes. They also demonstrated the absence of association between antibody response to BCG and a favorable clinical response.

In the present study no correlation was observed between CIC levels and the cellular type of leukemia: T-cell, B-cell or null-cell leukemia. No relationship was found between clinical hemorrhage and thrombocytopenia as other authors have reported [4]. Results correlated strongly with the clinical progression of the disease and development of high numbers of blasts.

In comparison the ^{125}I-C_1q binding assay is more discriminative than the PEG precipitation assay. Detection of CIC by the first method showed high values in induction and in relapse, and decreased levels in remission. Similar results were obtained by Carpentier et al. [6] applying the same method and also the Raji cell radioassay. Nevertheless, other authors [23] did not find a correlation between CIC levels obtained by the solid-phase bovine conglutinin test and clinical status in nonlymphatic leukemia. Clague et al. [8], using the C_1q radioiodinated assay did not observe any correlation between the presence of CIC and the prognosis in ALL, although they observed a strong correlation with a history of recent infection.

The PEG precipitation assay showed high CIC levels in induction only in 36% of the patients and about 20% in remission and in relapse phases. A possible explanation may be that different CIC detection methods may measure immune complexes of different nature, structure, and stability. The PEG precipiation assay may detect immune complexes of relative instability in comparison with those detected by the ^{125}I-C_1q binding assay. For instance, PEG immune complex values fall rapidly following repeated freezing and thawing [21] in gynecological malignancies. Another possibility, based on the report of Soltis and Hasz [26], is to use a final concentration of 2% PEG to obtain more discriminative results. They achieved a high degree of sensitivity between aggregated immunoglobulin and monomeric IgG.

The demonstration of complexed material with characteristics of precipitable immune complexes in ALL patients in the active stage, i.e., in induction and in relapse, should suggest the basis for studies on the prognostic usefulness of CIC.

Further investigations may facilitate the deciphering of the nature of the antigenic fraction and the antibodies involved in patients with ALL. Finally, studies related to the effect of CIC on interleukin production by mononuclear cells during progressive malignancies, as some authors have demonstrated in experimental animals [22], may allow a better understanding of other CIC effects.

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