Within the past 15 yr, many factors have contributed to a marked improvement in the prognosis of childhood acute lymphoblastic leukemia (ALL). Newer chemotherapeutic and support programs have resulted in survival of approximately 40%-50% of children with ALL for at least 5 yr.\(^1\)\(^3\) Despite these successes, it has become increasingly important to critically evaluate those children who have failed to respond to current treatment regimens. It is possible that unresponsive children with ALL represent subsets of this disease that are biologically distinct and, as such, require different therapeutic strategies. Insight into the heterogeneity of ALL has resulted from recent studies from several laboratories that have identified normal lymphocyte differentiation antigens and receptors on the surfaces of these neoplastic cells. Approximately 20% of children with ALL have lymphoblasts with surface receptors for sheep erythrocytes and T-cell antigens.\(^4\)\(^6\) The remaining 80% of children have lymphoblasts that lack B-cell surface characteristics, such as surface immunoglobulin, but do have la-like antigens.\(^5\)\(^9\)\(^2\)\(^3\) More importantly, it appears that patients with lymphoblasts of T-cell lineage have a much poorer prognosis than those with a reciprocal subset of non-T-cell lymphoblasts.\(^4\)\(^6\)

Earlier studies from this laboratory have demonstrated that three antisera, anti-HTA, anti-la (p23,30), and anti-CALLA, are particularly useful in defining distinct subsets of ALL cells.\(^7\)\(^10\) HTA antiserum was prepared against a T-cell variant of ALL, and the antiseraum reacts only with T-cell ALL cells, normal thymocytes, and circulating T cells. The la antiseraum was prepared against a glycoprotein complex (p23,30) purified from a human B-cell lymphoblastoid line.\(^11\) Anti-la reacts with B cells, B-cell lines, monocytes, and subsets of null cells destined to differentiate into B cells, myeloid cells, or erythrocytes.\(^7\)\(^9\)\(^12\)\(^13\) Antisera to the common ALL antigen (CALLA) was prepared against the lymphoblasts of an individual with HTA− la+ lymphoblasts. Details of the preparation and characterization of this antisera are reported elsewhere.\(^10\)

In this article, we describe the clinical characteristics and results of therapy of a homogeneously treated group of patients who have been followed for a median of 4 yr and in whom subsets of ALL were defined phenotypically by three cell surface markers: HTA, la, and CALLA.

**MATERIALS AND METHODS**

The diagnosis of ALL was established by two or more experienced morphologists using previously described criteria.\(^1\) Patients with lymphoma-like disease were included if the bone marrow contained greater than 25% lymphoblasts at the time of diagnosis.

One-hundred thirty-seven consecutive children, 20 yr of age and under, presenting to the Sidney Farber Cancer Institute or the Children’s Hospital Medical Center with previously untreated ALL were entered on the treatment program between October 1973 and May 1977. Informed consent was obtained from parents or guardians before therapy was begun. The results of treatment for this group have been previously reported.\(^1\) The median follow-up for this homogeneously treated group is 4 yr. The presenting clinical characteristics of 93 patients in whom immunologic cell surface marker studies were obtainable are listed in Table I. In the remaining 44 patients, cells could not be obtained for study. Most of these latter
patients were seen during the first year of the treatment program when marker studies and cryopreservation facilities were not readily available.

**Treatment Regimen**

Remission induction consisted of vincristine and prednisone. Asparaginase was used to effect early complete remission. Central nervous system prophylaxis consisted of whole brain irradiation using a linear accelerator to 2400 rad/1.3 fx/17 days and intrathecal methotrexate given within the same time span.16 Hereafter, intrathecal methotrexate was administered every 18 wk. Treatment in remission involved courses of Adriamycin, 6-mercaptopurine, vincristine, and prednisone given every 3 wk. After completion of a total cumulative Adriamycin dose of 450 mg/sq m of body surface area, the Adriamycin was replaced by parental methotrexate for the duration of treatment. Dosages of myelosuppressive agents were adjusted to permit administration of maximum doses.15 Therapy was electively discontinued after 30 mo of continuous complete remission.

**Cell Preparation**

Heparinized bone marrow samples were collected, and mononuclear cells were purified by Ficoll-Hypaque density sedimentation.16 Isolated mononuclear cells were cryopreserved using standard techniques and stored at -196°C with 5% DMSO and 5% fetal calf serum. In several patients, cells were tested immediately and were not frozen. There was no difference between surface markers of fresh or frozen cells. Cytocentrifuge smears of samples after Ficoll-Hypaque purification were used to verify the predominance of lymphoblasts in all samples subsequently analyzed for surface markers.

**Preparation of Antisera**

Rabbit T-cell antisera (HTA) were raised by intravenous injection of E-rosette positive, Ia negative-leukemic cells. HTA was made specific for peripheral blood T cells, thymocytes, and T-cell ALL by absorption with AB erythrocytes, cells from an autologous lymphoblastoid B-cell line, and cells from an allogeneic leukemic T-cell line, CEM.15 Anti-CALLA reacts specifically with cells from 80% of patients with HTA- Ia+ ALL and from some patients with chronic myelogenous leukemia in "blast crisis," but does not react with cells from patients with T-cell ALL or acute myeloblastic leukemia, or from leukemic T-cell or lymphoblastoid B-cell lines. Normal peripheral blood lymphocytes and bone marrow cells are also unreactive.

The survival curves were plotted according to the method of Kaplan and Meier.17 Analysis of the curves was carried out using the log rank statistic.21 A Cox proportional hazard regression model was used for a multivariate analysis of prognostic factors.22

**RESULTS**

With the exception of five patients whose lymphoblasts had unusual surface markers (see below), all HTA+ patients were Ia- CALLA-, and all Ia+ patients were HTA-. Therefore, for purposes of clarity, we will subsequently refer to HTA+ Ia- patients only as "HTA+," and to HTA- Ia+ patients only as "Ia+." The use of anti-CALLA will be added to the Ia+ group whenever necessary.

Representative fluorescence-activated cell sorter (FACS-I) printouts plotting fluorescence intensity versus cell number (for 40,000 cells analyzed) are illustrated for three patients with ALL in Fig. 1: Fig. 1A shows a patient with HTA+ ALL; Fig. 1B shows Ia+ ALL; and Fig. 1C shows Ia+ CALLA+ ALL.

Of the 93 patients who had bone marrow lymphoblasts characterized at the time of diagnosis, 88 could be divided into one of two classes: HTA+ or Ia+. Cells from 43 of the latter patients were available for testing with anti-CALLA. Presenting characteristics of these 88 patients by immunologic marker are shown in Table 1, and the results of treatment based on immunologic marker are shown in Table 2.

Five patients had unusual surface markers. One was HTA+ Ia+ CALLA+. He was a 9-yr-old who presented without a mediastinal mass or elevated WBC (defined herein as >25,000/cu mm). He remains disease-free at 4.5 yr and has been off all chemotherapy for 2 yr. Of the 4 patients who were HTA- Ia-, only 3 had enough cells available to assay for CALLA and all 3 were CALLA-. One patient was a 19-mo-old female who presented without a mediastinal mass or elevated WBC. She failed to enter complete remission and died soon thereafter. Another of these patients was an 11-mo-old male who presented without a mediastinal mass but with a WBC of 93,000/cumm. He entered complete remission but was withdrawn from treatment to be taken to a faith healer. The other two children presented without mediastinal masses or elevated WBCs. Both remain disease-free for 1.5 and 3.5 yr, respectively, the latter off treatment for 1 yr.
Fig. 1. (A–C) Indirect immunofluorescence assay of leukemic cells and anti-HTA, anti-la, and anti-CALLA using the fluorescence-activated cell sorter for analysis. Forty-thousand cells were counted in each sample and presented as a histogram of number of cells versus intensity of fluorescence for each antigen. Normal rabbit serum is used as a negative control in each analysis. (A) Leukemic cells are anti-HTA positive, anti-la negative. (B) Leukemic cells are anti-HTA negative. anti-la positive. (C) Leukemic cells are anti-HTA negative, anti-la positive, anti-CALLA positive.
As shown in Table 1, the HTA+ group were older, had a male predominance, and most had mediastinal masses and elevated WBCs. As shown in Table 2, 10 of the 11 patients entered complete remission. Six of the patients relapsed between 3 and 23 mo, with a median time to relapse of 12 mo (Fig. 2). Primary sites of relapse were the bone marrow in two patients, meninges in one, testes in one, testes and meninges in one, and bone marrow, testes, and meninges in one.

Three of the 10 complete remitters were withdrawn from this treatment protocol while in complete remission when it became apparent that HTA+ individuals were responding poorly to the treatment program. Leukemic cells in 10 of the 11 HTA+ patients were tested for the formation of E-rosettes, and all were found positive.

The 77 children with la+ lymphoblasts had a median age of 5 yr, an equal male-to-female ratio, and presented without mediastinal masses (Table 1). Seventy-three entered complete remission.

Of the 73 complete remitters in the Ia+ group, one was lost to followup, one died in remission, and 29 relapsed (Fig. 2). Twenty-four of the relapses were in the bone marrow only and occurred between 9 and 48 mo after entering complete remission. Three patients developed primary meningeal relapses. One child had originally presented with central nervous system involvement, another had received cranial irradiation through inadequate ports, and the third child’s nervous system relapse could not be explained. Simultaneous bone marrow and meningeal relapse occurred in one patient 44 mo after entering complete remission. The only testicular relapse occurred in a patient who had been disease-free for 33 mo. Of the children who relapsed, 2 were under age 2 yr at the time of diagnosis, 6 were over age 9 yr, and 10 had presented with elevated WBCs. Forty-four of the 73 complete remitters completed 30 mo in remission and have been off chemotherapy for 1–37 mo (median time off treatment is 21 mo); 11 of these relapsed after elective cessation of chemotherapy. The difference in disease-free survival between the HTA+ and Ia+ patients shown in Fig. 2 is statistically significant using the log rank test ($p = 0.0004$).

Of the 77 Ia+ patients, 43 were tested for CALLA; 36 were CALLA+ and 7 were CALLA− (Table 2). Thirty-four of the CALLA+ patients and all 7 of the CALLA− patients entered complete remission. Figure 3 shows the disease-free survival for these 41

### Table 2. Results of Treatment in 88 Patients: Evaluation by Cell Surface Markers

<table>
<thead>
<tr>
<th></th>
<th>HTA+</th>
<th>Total Ia+</th>
<th>CALLA+</th>
<th>CALLA−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number evaluable</td>
<td>11</td>
<td>77</td>
<td>36</td>
<td>7</td>
</tr>
<tr>
<td>Number complete</td>
<td>10</td>
<td>73</td>
<td>34</td>
<td>7</td>
</tr>
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<td>Withdrawals or</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
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<tr>
<td>remission deaths*</td>
<td></td>
<td></td>
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<tr>
<td>Duration of remission</td>
<td>(See Fig. 3)</td>
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**Relapses**

- Number: 6, 29, 15, 3
- Bone marrow only: 2, 24, 12, 3
- Meninges only: 1, 3, 2, 0
- Marrow and meninges: 0, 1, 0, 0
- Testes only: 1, 1, 1, 0
- Testes and meninges: 1, 0, 0, 0
- Testes, meninges and marrow: 1, 0, 0, 0

*Three HTA+ patients were removed from protocol in remission at 11+, 11+ and 8+ mo (see text). One Ia+ CALLA+ patient died in remission at 1+ mo, one Ia+ CALLA− unknown patient died in remission at 16+ mo, and one Ia+ CALLA− patient was lost to follow-up at 1 mo. Patients who were removed from protocol or lost to follow-up are considered incomplete (censored) observations in the life table analyses. Patients who died in remission are considered failures at the time of death.

Fig. 2. Disease-free survival for 73 Ia+ and 10 HTA+ patients. The difference is statistically significant ($p = 0.0004$).

Fig. 3. Disease-free survival for 41 Ia+ patients who were also tested for CALLA. Within the Ia+ group, the difference between CALLA+ and CALLA−patients is not statistically significant.
patients. Although with this small number of patients the results are not statistically significantly different within the Ia+ groups, these data are suggestive that CALLA positivity is a favorable prognostic factor.

Patients with elevated WBC and those who are either older or very young have traditionally been considered to have an adverse prognoses. Table 3 shows the projected median duration of disease-free survival by WBC and age for our complete remitters in both the HTA+ and Ia+ groups. As expected, a high proportion of HTA+ patients fall into the traditionally poor prognostic group, and our data show a trend toward worse prognosis that follows traditional guidelines. A proportional hazard regression model,23 has been used to ascertain the relative prognostic importance of the HTA as well as traditional factors. While neither age nor WBC are significant prognostic factors alone, when both factors are considered together (resulting in the four categories as shown in Table 3), the differences in outcome are statistically significant ($p = 0.001$). When the HTA factor is added to this model, the fit of the model is significantly improved ($p = 0.014$; using twice the difference in maximum log likelihood). Thus, even when age and initial WBC are accounted for in this fashion, HTA+ patients do much worse than Ia+ patients.

Figure 4 shows the disease-free survival curves for Ia+ patients by age category and initial WBC. In contrast to the significant difference seen for the total group of 83 patients ($p = 0.001$ using the log-rank test), the difference among the curves shown in Fig. 4 for the Ia+ patients is not statistically significant. However, there did appear to be a trend along traditional lines that may make it possible to identify a poor prognostic group within the Ia+ population. Identification of such a group by the use of cell surface marker as well as traditional prognostic factors will allow a more precise definition of subgroups.

**DISCUSSION**

The present studies confirm and extend previous observations that T-cell (HTA+) ALL patients respond poorly to current therapeutic regimens and indicate that serologic methods provide a means for prompt and reliable diagnosis. The HTA antisera define a distinct subpopulation of lymphocytes and is reactive with thymocytes, peripheral T cells, and T-cell leukemic blasts, but is unreactive with B lymphocytes and normal myeloid and erythroid cells. Although patients with leukemia identified as HTA+ in this series were also E-rosette positive, we and others have noted instances where leukemic cells were HTA+ and E-rosette negative. We, therefore, do not routinely use the E-rosette test to define T-cell leukemias.

The Ia antigen, in contrast, is found on lymphoblasts of approximately 80% of children with ALL, in normal B cells, monocytes, myeloid, and erythroid progenitor cells, and on a null cell subset destined to differentiate into B cells. This antigen is serologically and biochemically distinct from HLA antigens and is analogous to the murine Ia antigens and other B-cell alloantigens, which have been shown to play a major role in the stimulation of the allogeneic mixed lymphocyte response.

From the standpoint of differential diagnosis, the Ia antiserum is able to confirm the diagnosis of T-cell ALL, since the Ia antigen is normally absent on T-cell surfaces. In contrast, most other forms of ALL and acute myelogenous leukemia possess the Ia antigen. Thus, the HTA and Ia antigens define reciprocal subsets of ALL. Moreover, we can further define the Ia+ subset with the CALLA antiserum; cells of patients with Ia+ ALL may be CALLA+ or CALLA-. Patients whose lymphoblasts were CALLA- appeared to do poorly when compared with
CALLA+ patients; this finding is analogous to the experience of others.\textsuperscript{5}

The relative prognostic importance of cell surface markers as compared to traditional clinical prognostic factors, such as age and WBC at the time of diagnosis, has been explored. It is apparent that much overlap exists between patients who are HTA\textsuperscript{+}, older, and have elevated WBCs. Clinical heterogeneity also exists within immunologically homogeneous patient groups. Treatment programs must be based on the most discriminating prognostic factors. For example, 3 of 11 HTA\textsuperscript{+} patients did not have elevated WBCs at the time of diagnosis, and 4 of 11 did not have radiographically apparent mediastinal masses.

It must also be recognized that methods of immunologic classification will continue to improve. Indeed, subsets of HTA\textsuperscript{+} patients have already been recognized, as have clinical and prognostic variability within such subsets.\textsuperscript{32,33}

Our multivariate analysis of the results, presented in Table 3, demonstrates that the combination of presenting ages and initial WBC has prognostic significance in the total patient group. In addition, HTA type has prognostic significance even when traditional clinical factors are controlled in the analysis. The overall prognostic value of age and WBC in our Ia\textsuperscript{+} patients using this treatment program is not statistically significant (Fig. 4). However, subgroups of Ia\textsuperscript{+} patients who have poorer prognoses may yet be identified. These subgroups may be distinguished not only on the basis of clinical findings, but more specifically on the basis of immunologic markers. Therapeutic results must be viewed in the context of a particular treatment program. Future treatment programs must consider possible biologic differences in the leukemias, such as those manifested by immunologic markers, as well as traditional factors.

Why is conventional therapy unable to maintain continuous complete remission in the HTA\textsuperscript{+} patients? Suboptimal chemotherapy may in part contribute to the failure of this group. Evaluation of chemotherapy regimens in experimental leukemia systems shows that drugs effective against thymus-derived AKR leukemia are frequently different from those effective in conventional mouse lymphoid leukemia systems.\textsuperscript{34} Specifically, antimetabolites such as methotrexate and 6-mercaptopurine, both highly active in conventional, non-T-cell leukemia, are relatively inactive in the AKR system. On the other hand, cyclophosphamide and cytosine arabinoside are more active against T-cell leukemias than against the conventional models.

Distribution and migration of the HTA\textsuperscript{+} lymphoblasts may be different from Ia\textsuperscript{+} lymphoblasts, resulting in sequestration of cells in extramedullary sites and escape from chemotherapy in the former group. Studies in mice demonstrated that purified T cells migrate to the skin and other soft tissues, whereas B cells invariably circulate and migrate into lymph nodes and spleen.\textsuperscript{35} This tropism would account for the more successful control of extramedullary disease in Ia\textsuperscript{+} leukemia and provide an explanation for the frequency of and sites for extramedullary relapse in the HTA\textsuperscript{+} population.

In this treatment program 67% of the relapses in the HTA\textsuperscript{+} patients and 17% of the relapses in Ia\textsuperscript{+} patients were at extramedullary sites. With the exception of two explainable meningeal relapses (inadequate irradiation and presentation with preexisting nervous system involvement), the incidence of central nervous system relapse was 50% for HTA\textsuperscript{+} and 7% for Ia\textsuperscript{+} patients who relapsed. Three of the four testicular failures were in the HTA\textsuperscript{+} group. These patients' relapses occurred early in the course of therapy, whereas the Ia\textsuperscript{+} patient's relapse occurred after elective cessation of chemotherapy. In part, these data are in accord with a previous report that suggested a temporally bimodal pattern of testicular relapse.\textsuperscript{36} In contrast to recent reports of an increased incidence of testicular relapse after cessation of therapy,\textsuperscript{36-39} we have found only one such patient. The generally unsuccessful treatment of T-cell leukemia and its pattern of early relapse suggest that the late testicular relapses reported by others occur mainly in patients with non-T-cell disease. The difference between our experience and that of others is probably due to differences in treatment programs. The major innovation in our program is the early and intensive use of adriamycin. Our results are of importance because they indicate that prophylactic gonadal irradiation may be unnecessary in Ia\textsuperscript{+} patients. Moreover, this further emphasizes that the course of leukemia must be viewed in the context of particular therapeutic programs, as well as that of cell surface markers.

The precise cellular derivation of the Ia\textsuperscript{+} cells is unknown; however, the presence of the Ia antigen and the absence of T-cell antigens suggest that these leukemic cells may be derived from the B-cell lineage. The finding of intracytoplasmic IgM in a significant percentage of non-T leukemia lymphoblasts is further evidence for this hypothesis.\textsuperscript{40,41} Since the Ia antigens are critical in the initiation of a mixed lymphocyte response and in the generation of cytotoxic T lymphocytes, the absence of these antigens on the HTA\textsuperscript{+} cells may also help to explain the poor prognosis of these patients.

In summary, we have described leukemia-associated antigens, HTA, Ia and CALLA, and their
IMMUNE MARKERS IN CHILDHOOD ALL

distribution in a childhood leukemia population; HTA+ (12%), IA+ (88%), and of those who are IA+, 84% are CALLA+ and 16% CALLA−. We have confirmed the finding that patients with T-cell disease have a worse prognosis than non-T-cell patients in a large population receiving an identical treatment program. Current treatment protocols appear to be highly effective in the subset of non-T-cell patients who are CALLA+. Improved regimens are required for the treatment of HTA+ patients and possibly for IA+ CALLA− patients as well. Immunologic characterization of distinct subsets of ALL such as these should allow for the refinement of treatment programs specific for each subset.

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Cell surface antigens: prognostic implications in childhood acute lymphoblastic leukemia

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