Many human lymphocyte cell lines have been established from subjects with a variety of hematopoietic disorders as well as from normal individuals. Most of these cell lines have properties of bone marrow-derived lymphocytes, or B cells (1-5): immunoglobulins can be detected in their cytoplasm, on their surface membranes, or secreted into the surrounding medium. Another nearly universal feature is the presence in the cells of Epstein-Barr virus (EBV) or EBV antigens (5-8).

We have recently shown (9) that cell lines CCRF-CEM and HSB-2, derived from two children with leukemia secondary to lymphosarcoma, have T-cell properties, as do tumor cells obtained directly from other children with lymphoblastic lymphoma. These two lines, extensively studied by Adams, Foley, and their colleagues (10-13), possess characteristics of malignant lymphoblasts, not only by their ability to multiply indefinitely in vitro, but also by their oncogenic behavior when injected into untreated newborn hamsters.

The only other cell lines described with T-cell properties are the "MOLT" cell lines isolated and characterized by Minowada, et al. (14). These four lines, derived from a 19-year old male with acute lymphoblastic leukemia, lack EBV and EBV antigens (15). Because EBV or EBV antigens have been found in every B-cell line so studied, the possibility that T-cell lines derived from malignant lymphoblasts lack EB virus requires further investigation. This report describes studies of cell lines CCRF-CEM and HSB-2 which, in addition to further demonstrating that they share many properties with normal peripheral blood T cells, indicate that they are EB virus-negative.

Materials and Methods

Cell Cultures.—The sources and methods of maintaining cultures of cell lines CCRF-CEM, HSB-2, CCRF-SB, and Raji have been previously reported (9). Cell line P3HR1 (16) was maintained in a similar manner.

T- and B-Cell Characterization.—The methods for detection of erythrocyte-binding lym-
phocytes, complement receptor-bearing lymphocytes, and surface immunoglobulin-bearing lymphocytes were as previously described (9).

Antisera.—Equine antihuman thymocyte gamma globulin (Upjohn Co., Kalamazoo, Mich.) was dialyzed extensively against 0.15 M NaCl, heat inactivated at 56°C for 30 min, and absorbed with pooled human type AB-positive erythrocytes. Rabbit antisera to lymphocyte cell lines were raised by twice injecting 10⁸-10⁹ cells i.v. at 10-day intervals and bleeding 7-10 days after the last injection. Serum was heat inactivated and stored at -20°C.

Inhibition of Rosette Formation.—Diluted antiserum (0.05 ml) was added to aliquots of cells (0.25 ml) at 10⁶ cells/ml of Hanks’ basal salt solution. After 15 min at room temperature, 0.5% washed sheep erythrocytes (0.25 ml) were added and rosette formation was determined (9).

Detection of EBV Antigens.—Previously described methods to detect EB viral capsid antigen (VCA) (17), and EB viral nuclear antigen, EBNA (18), were employed using fluorescein isothiocyanate conjugated (FITC) goat polyvalent antihuman Ig and FITC goat antihuman β1C/β1A globulin obtained from Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif. Two human sera containing EB viral capsid antibody at dilutions of 1:80 and 1:360 respectively, and two human sera without viral capsid antibody at 1:5 dilutions were selected. The former two sera contain antibody directed against EBNA; the latter two do not. The VCA test antigen was acetone-fixed P3HR1 cells; the EBNA test antigen was acetone-fixed Raji cells.

RESULTS

Table I shows that cell lines CCRF-SB, Raji, and P3HR1, like most previously described lymphocyte cell lines, are B cells. They possess complement receptors and surface immunoglobulin. In contrast, cell lines CCRF-CEM and HSB-2 are T cells. They form rosettes with sheep erythrocytes and lack complement receptors and surface immunoglobulin.

Examination of various aspects of rosette formation reveals many additional similarities between these T-cell lines and normal peripheral blood T cells (Table II): More rosettes form after incubation at 4°C than after incubation at 37°C, and rosette formation occurs to varying degrees, with erythrocytes from different species. Of note is the observation that, like normal human T cells, under appropriate temperature conditions, both CCRF-CEM and HSB-2 form rosettes with human erythrocytes. In all of these instances, binding of erythrocytes to the cell surface is weak since even brief, but vigorous agitation totally disrupts the rosettes.

A comparison of the ability of antisera raised against T- and B-cell lines and against human thymocytes to inhibit in vitro rosette formation with sheep erythrocytes is shown in Table III. Rabbit antisera to the T-cell line CCRF-CEM as well as equine antihuman thymocyte gamma globulin inhibit rosette formation with sheep erythrocytes by normal peripheral blood T cells as well as by both T-cell lines. In contrast, rabbit antisera to the B-cell lines CCRF-SB and Raji have no effect on rosette formation even though they possess the same degree of reactivity as antithymocyte serum and antisera to CCRF-CEM

2 Kaplan, J. Human T cells from rosettes with autologous and homologous human erythrocytes. Manuscript in preparation.
TABLE I
T- and B-Cell Markers on Cells from Established Lymphoid Cell Lines

| Cell line   | EL* | CRL* | SIg* |
|-------------|-----|------|------|
| CCRF-CEM    | 51  | 0    | 0    |
| CCRF-HSB-2  | 43  | 0    | 0    |
| CCRF-SB     | 0   | 2    | 90   |
| Raji        | 0   | 63   | 96   |
| Peripheral blood of eight normal adults | 60 ± 8† | 17 ± 4 | 25 ± 5 |

* Average of three determinations of each cell line. EL, erythrocyte-binding lymphocytes; CRL, complement receptor lymphocyte; and SIg, surface immunoglobulin lymphocytes. † Mean ± SD.

TABLE II
Rosette Formation by Lymphocyte Cell Lines at Different Temperatures and with Erythrocytes from Different Species

| Cells                      | % rosettes formed with sheep erythrocytes after incubation at: | % rosettes formed at 4° with erythrocytes from: |
|----------------------------|---------------------------------------------------------------|--------------------------------------------------|
|                            | 4°    | 37°  | Dog | Goat | Horse | Hamster | Human* |
| Peripheral blood           | 55    | 1    | 11  | 14   | 11    | 0       | 8      |
| CCRF-CEM                   | 31    | 15   | 25  | 11   | 5     | 0       | 5      |
| CCRF-HSB-2                 | 26    | 7    | 57  | 24   | 25    | 5       | 5      |
| Raji                       | 0     | 0    | 0   | 0    | 0     | 0       | 0      |

* Read at 4°.

TABLE III
The Effect of Antiserum to Human Thymocytes and to Lymphocyte Cell Lines on Sheep Erythrocyte Binding by Cell Lines HSB-2 and CEM

| Serum               | Dilution | Inhibition of erythrocyte binding* by: |
|---------------------|----------|--------------------------------------|
|                     |          | CCRF-CEM | HSB-2 |
| Anti-CCRF-CEM       | 1:60     | 100      | 100   |
| Anti-CCRF-SB        | 1:60     | 9        | 0     |
| Anti-Raji           | 1:60     | 0        | 0     |
| Anti-human thymocyte| 1:100    | 100      | 100   |

* % inhibition of rosette-forming cells (RFC) = \(100 \times (\% \text{RFC}_{\text{control}} - \% \text{RFC}_{\text{serum}}) / \% \text{RFC}_{\text{control}}\).

when assayed for cytotoxicity to peripheral blood lymphocytes and cell line lymphoblasts. These findings indicate that the T-cell lines, but not the B-cell lines, share a common surface antigen with normal peripheral blood T cells. EB virus or EB viral antigens exist in most lymphocyte cell lines. Since the
T-cell properties of cell lines CCRF-CEM and HSB-2 make them uniquely different from most other established lymphocyte cell lines, they were examined for the presence or absence of EB viral antigens. The results of testing for VCA and for EBNA are shown in Table IV.

As previously reported (19), cell line P3HR1 shows a positive reaction for VCA consisting of intense bubbly fluorescence seen in approximately 5% of the cells, while the VCA-negative cell line Raji shows only weak background fluorescence. A similar negative VCA reaction is seen with cell lines CCRF-SB, HSB-2, and CCRF-CEM. When tested for EBNA, cell line Raji, as previously demonstrated (18) exhibits bright, feathery nuclear fluorescence in over 90% of the cells. A similar positive reaction is seen with the B-cell line CCRF-SB. In contrast, the T-cell lines CCRF-CEM and HSB-2 contain no positively stained cells. Their nuclei are uniformly dark against weak background cytoplasmic autofluorescence.

**DISCUSSION**

Human T cells form nonimmune rosettes with sheep erythrocytes (20, 21) whereas human B cells have complement receptors (22) and surface immunoglobulins (23). By use of these markers, cell lines CCRF-CEM and HSB-2 resemble "normal" human T lymphocytes. They bind sheep erythrocytes and erythrocytes of certain other species, this binding is inhibited to a greater degree by heterologous antiserum to T-cell lines than by heterologous antiserum to B-cell lines, and they lack complement receptors and surface immunoglobulin.

However, while they possess features in common with normal T cells, evi-

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**Table IV**

Testing of Lymphocyte Cell Lines for EB VCA and EBNA

| Cells          | Reference serum (EBV antibody*) | Cells staining for: |
|----------------|---------------------------------|--------------------|
|                |                                 | VCA               |
|                |                                 | EBNA              |
| P3HR1          | Positive                        | 5                 |
|                | Negative                        | 0                 |
| Raji           | Positive                        | 0                 |
|                | Negative                        | 0                 |
| CCRF-SB        | Positive                        | 0                 |
|                | Negative                        | 0                 |
| CCRF-HSB-2     | Positive                        | 0                 |
|                | Negative                        | 0                 |
| CCRF-CEM       | Positive                        | 0                 |
|                | Negative                        | 0                 |

* All sera diluted 1:10.
ence suggests that both of these lines represent malignant T cells. Both CCRF-CEM and HSB-2 originated from peripheral blood of two children with lymphoblastic lymphoma (12). Tumor cells obtained directly from four children with lymphoblastic lymphoma have been shown to have T-cell properties (9). No lymphocyte cell line with T-cell properties has yet been established from a normal individual. Adams and his co-workers have studied the ability of these and other cell lines to cause tumors in hamsters. They have shown (13) that cell lines CCRF-CEM, HSB-2, and a third cell line from a child with lymphoblastic lymphoma, H-HM-1, induce tumors in untreated newborn hamsters which progress to leukemia. The tumor cells do not produce immunoglobulin. No other cell lines tested cause tumors in untreated hamsters. However, after antithymocyte serum treatment of animals, a variety of lymphocyte cell lines, including cell lines CCRF-SB and cell lines from normal individuals, do cause tumors in newborn hamsters. These do not progress to leukemia. Cells from these tumors, however, like B cells, produce human immunoglobulin. These findings, taken together, support the concept that cell lines CCRF-CEM and HSB-2 represent malignant T lymphocytes.

In contrast, cell line CCRF-SB, which was established from the same patient as cell line HSB-2, has B rather than T-cell properties. CCRF-SB does not form rosettes with sheep erythrocytes and does have membrane complement receptors and surface immunoglobulins. All lymphocyte cell lines obtained from normal individuals are B cells (1, 2, 5), therefore cell line CCRF-SB probably originated from a normal, albeit EBV-infected, B-lymphocyte population which co-existed with the malignant T lymphoblasts from which cell line HSB-2 originated.

EB virus apparently only infects B cells: Burkitt's lymphoma tumor cells contain EB viral antigens and are B cells; B cells, but not T cells, have surface receptors for EB virus (15); and finally, evidence of EB virus or viral antigens is found in all B-cell lines but not, as shown here, in T-cell lines. Like the previously described MOLT T-cell lines (15), CCRF-CEM and HSB-2 are EB virus-negative, even when examined for the EBNA which has been found in all cell lines possessing EBV genome (18).

That EB virus can induce the establishment of lymphocyte cell lines from fetal lymphoid tissue, and that establishment of lymphocyte cell lines from normal individuals can only occur if the donors have serological evidence of prior EBV infection, has suggested that EB virus infection is a prerequisite for sustained in vitro lymphocyte proliferation. The EBV-negative T-cell lines CCRF-CEM, HSB-2, and MOLT appear to be exceptions to this rule.

Factors other than EBV genome must be responsible for their continuous in vitro growth. HSB-2 was originally established from leukemic cells hetero-transplanted into newborn hamsters. However, passage through hamsters is not a necessary condition for establishment of T-cell lines since CCRF-CEM and the MOLT cell lines were established directly from peripheral blood.
samples. Since all the known T-cell lines originate from subjects with leukemia while no T-cell lines have yet been established from normals, and since cell lines CCRF-CEM and HSB-2 both possess unique in vivo oncogenic behavior in hamsters, their continuous in vitro growth probably reflects their origin from malignant tumor cells. Perhaps they possess genetic material of a virus which, like EB virus (6, 24–26), can induce both long term in vitro cell growth as well as in vivo tumor growth. If so, the ready availability of these malignant T-lymphocyte cell lines should facilitate the search for a human leukemia-lymphoma virus.

SUMMARY
Two lymphoblastoid lines, CCRF-CEM and HSB-2, with properties of malignant cells, derived from children with leukemia secondary to lymphosarcoma, have T-cell properties and lack Epstein-Barr virus antigens.

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