L2C Guinea Pig Lymphatic Leukemia:
A "B" Cell Leukemia

By E. M. Shevach, L. Ellman, J. M. Davie, and I. Green

Lymphoid cells of the immune system can be divided into two functional compartments. The thymus derived population, "T" cells, is responsible for cell mediated immunity. The bone marrow derived population, "B" cells, is responsible for antibody production. Although these two populations are functionally different, it has not yet been possible to distinguish them morphologically. Recent experimental work in the mouse has shown that the B cells bear easily detectable immunoglobulin. The T cells can be distinguished by the isoantigen, theta. The B or T cell origin of the lymphocytes of human or animal leukemia has received little attention. In the present study, we have examined the functional and morphologic properties of a guinea pig lymphatic leukemia L2C. L2C cells secrete Tg immunoglobulin and also bear this immunoglobulin on their surface. L2C cells have the recently described lymphocyte receptor for antigen-antibody-complement complexes (found on normal B lymphocytes). Finally, the L2C cell fails to be stimulated in vitro by mitogens capable of stimulating thymus-derived lymphocytes. Thus, the L2C cell appears to be of B lymphocyte origin. The availability of a large number of pure B lymphoid cells will provide a useful tool for the study of the cellular receptors of lymphoid cells and for the preparation of antisera specific for the T cell and B cell populations. The application of the techniques described in this paper to classify other lymphoid neoplasms as to their T or B cell origin may lead to both theoretic and therapeutic advances.

OVER THE PAST DECADE, evidence has accumulated from experimental studies in mammals and birds and from the investigation of certain human diseases such as Bruton type agammaglobulinemia and thymic aplasia (the DiGeorge syndrome) that the lymphoid cells of the immune system can...
be divided into two functional compartments. One population of lymphoid cells comes under the influence of the thymus and is termed thymus derived (T cells); this population is responsible for cell mediated immunity and graft rejection. In the chicken, the other population of cells that is responsible for antibody production, comes under the influence of the bursa of Fabricius. In mammals, these cells probably pass directly from the bone marrow to peripheral lymphoid organs and are termed bone marrow derived lymphoid cells (B cells).

Although these two populations of cells have been shown to have markedly different biologic functions, it has not been possible as yet to distinguish them solely by morphology. However, Raff, using the immunofluorescent technique in the mouse, has recently identified a population of cells with surface immunoglobulin. These cells are absent from the thymus, and the percentage of them in lymph nodes and spleen can be increased by treatment of the animal with antilymphocyte serum or by thymectomy. It has therefore been suggested that these immunoglobulin-bearing cells belong to the thymus independent or B population of cells. Another population of lymphocytes can also be distinguished by the cytotoxic and immunofluorescent techniques because of the presence of the isoantigen theta. Theta-bearing cells make up 95%-100% of thymocytes and from 35%-55% of spleen cells. The population of theta positive cells in spleen and lymph nodes is markedly reduced by adult thymectomy, followed by lethal irradiation and bone marrow reconstitution. Hence, the theta isoantigen is the marker for most of the thymus-derived cells in the mouse.

Although the existence of the B and T cell lymphocyte populations in experimental animals is accepted by most immunologists, the bone marrow or thymic origin of the lymphoid cells of human and animal leukemias and lymphomas has received little attention. As a model for this type of investigation of leukemias, we have chosen a leukemia in inbred strain two guinea pigs. This leukemia, L2C, arose spontaneously in one animal in the colony maintained at the National Institutes of Health and has been maintained by serial passage in inbred strain two animals for over 17 yr. It fails to grow readily in inbred strain 13 animals or in random bred guinea pigs. A viral etiology of the leukemia had been proposed but has not been confirmed. Recent studies from our laboratory have demonstrated the presence of a tumor specific transplantation antigen (TSTA) on L2C cells by immunization protection tests.

In the present paper, evidence will be presented that the L2C guinea pig leukemia has multiple properties characteristic of the bone marrow-derived, B cell population. The approach used in this study, when applied to human lymphoid neoplasms, may also reveal that some are clearly derived from the B or T cell populations. This classification of human lymphoid tumors may lead to both theoretical and therapeutic advances.

MATERIALS AND METHODS

Inbred strain 2 guinea pigs were obtained from the rodent production section, National Institutes of Health. The animals were inoculated with one million L2C cells intradermally.
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Three weeks after inoculation, when the animals appeared moribund and had white blood cell counts greater than 300,000/cu mm, they were exsanguinated by cardiac puncture into a heparinized syringe. Red blood cells were sedimented by the addition of one-third volume of 3% gelatin in saline. The buffy coat containing the L2C cells was removed, and the cells washed three times in serum-free medium. Normal strain 2 lymph node cells were obtained from animals that had been injected in the foot pads with complete Freund's adjuvant, which was used to cause hypertrophy of the draining nodes.

Specific Antisera

Rabbit anti-guinea pig Tα, prepared by immunizing rabbits with purified heavy chain obtained from Tα immunoglobulin. The method of antibody purification and the specificity of this antibody have been previously described.8 This antibody precipitated only Tα heavy chains and failed to precipitate T1 immunoglobulin or light chains, as detected by immunoelectrophoresis and double diffusion in agar. The rabbit anti-guinea pig T1, anti-guinea pig L chain, and anti-guinea pig Fab were gifts of Dr. V. Nussenzweig (New York University School of Medicine). Rabbit anti-guinea pig IgM and rabbit anti-guinea pig colostral IgA were gifts of Dr. R. Asofsky (National Institutes of Health). Goat antirabbit IgG was kindly supplied by Dr. John Robbins (National Institutes of Health). A strain 13 antistrain 2 antihistocompatibility antiserum was prepared as previously described.9

Immuno fluorescent Studies

Rabbit anti-gpα, rabbit anti-gp Fab, and goat anti-rabbit IgG were conjugated with fluorescein isothiocyanate and passed over sephadex G-25 and DEAE columns according to the technique of Wood et al.10 Both indirect and direct staining were performed in the living state according to the technique of Moller.11 For indirect staining, 0.1 ml of a cell suspension containing 10 million cells in Hanks Balanced Salt solution (HBSS) was incubated at room temperature with an equal volume of the appropriate antiserum diluted 1:10. The cells were then washed three times with HBSS and resuspended in 0.1 ml of medium; 0.1 ml of the fluoresceinated antiserum was then added, and the suspension incubated at 4°C for 30 min. The cells were again washed three times and suspended in medium. One drop of the suspension was then examined under a vaseline-lined cover slip. At times, smears of the cell suspension were examined after sedimentation by a cytocentrifuge. When the direct fluorescent technique was used, the initial incubation at room temperature was omitted, and the cells were incubated directly with the fluoresceinated antiserum at 4°C. A Leitz fluorescent microscope with a high pressure mercury lamp (HB 200) was used for all examinations.

In Vitro Synthesis of Immunoglobulin

Five samples of 10 million L2C cells were washed three times in a tissue culture medium lacking lysine and supplemented with penicillin (Medium 320, NIH media Unit). The cells were suspended in 1 ml of this medium in 12 X 75 mm plastic tubes, and 0.1 ml of 5% ovalbumin and 0.1 ml of 14C-lysine (10 μCi, specific activity 312 Ci/mole, Amersham Searle, Des Plaines, Ill.) were added. The cultures were incubated for 14 hr and then centrifuged at 10,000 g for 20 min. The combined supernatants (5 ml) were pooled and dialyzed against 0.015 M NaCl for 24 hr. The culture fluid was then lyophilized and reconstituted in 0.5 ml of distilled water. Microimmunoelectrophoresis was performed using the technique of Scheidegger.12 Normal guinea pig serum or guinea pig colostrum was added as a carrier. Radioautography of the immunoelectrophoretic plates was performed according to the method of Hochwald et al.13

Detection of Lymphocyte Complement Receptor on L2C and Lymph Node Cells by Rosette Formation with EAC

One milliliter of Veronal-buffered saline (VBS)15 containing 5 X 10⁸ sheep red blood cells was incubated with an equal volume of rabbit anti-Forsman antibody diluted 1:500 in VBS. After 30-min incubation at 37°C, the RBC were washed twice with VBS and...
then stored at 4°C. Antigen-antibody-mouse complement complexes (EAC) were prepared by adding an equal volume of fresh mouse serum diluted 1:10 in VBS to the RBC suspension. Antigen-antibody complexes (EA) were prepared by adding an equal volume of fresh mouse serum diluted 1:10 in 0.01 M EDTA to the RBC suspension. These cell suspensions were incubated at 37°C for 30 min and then washed once in VBS (EAC) or EDTA (EA). After a second wash in VBS, both suspensions were reconstituted to a concentration of 1 X 10^8 RBC/ml in RPMI-1640 (Gibco, Grand Island, N.Y.). Four-tenths milliliter of the lymphoid cell suspension (2 X 10^6 cells/ml) to be tested was added to 0.4 ml of the EAC or EA suspensions. At times, the incubations were carried out in the presence of the 0.01 M EDTA to inhibit the binding of EAC to the complement receptor on monocytes.14 The mixtures were incubated at 37°C for 30 min with gentle agitation on a horizontal rotator. The number of lymphocytes with three or more RBC attached to the cell surface (rosettes) were enumerated in a hemacytometer. The total WBC count on an aliquot of the same sample was determined after lysis of the RBC with 2% acetic acid, and the per cent of rosette-forming cells was calculated.

Response to Mitogens

Two million L2C cells or strain 2 lymph node cells were cultured in 1 ml of minimal essential medium (Eagle) supplemented with L-glutamine (300 µg/ml), penicillin (100 U/ml), streptomycin (100 µg/ml), and 10% normal strain 13 serum. The appropriate concentration of mitogen was added in 0.1 µl. The cultures were performed in triplicate in plastic 12 X 75 mm tubes at 37°C in a humid atmosphere of 5% CO2 in air. After 28 hr, 1 µCi of 3H-thymidine (specific activity, 6.8 Ci/mole; New England Nuclear, Boston, Mass.) was added and the cultures continued for 18 hr. The amount of 3H-thymidine incorporated into cellular DNA was measured by the method of Robbins et al.16 using Millipore filters and standard liquid scintillation counting techniques. The mitogens used were purified phytohemagglutin (Wellcome Research Laboratories, Beckenham, England), concanavalin A (Nutritional Biochemicals, Cleveland, Ohio), and Pokeweed mitogen (Gibco, Grand Island, N. Y.).

RESULTS

Immunofluorescent Staining of L2C Cell by Antiimmunoglobulin

As can be seen from Table 1 and Fig. 1, the L2C cell had a diffuse speckled appearance when stained indirectly by rabbit anti-gp L chain, anti-Fab, and anti-λ. The anti-Fab reagent could stain the cell directly; the anti-κ and anti-L chain reagents were positive only when used as a middle layer in the indirect technique, followed by fluoresceinated goat anti-rabbit IgG. This difference is probably due to variation in the potency of the different antisera. In any given preparation, from 90%–100% of the L2C cells showed fluorescent staining (Fig. 1). In contrast, normal strain two lymph node cells when stained by the anti-κ reagent or by the anti-Fab reagent revealed only 30%–40% positive cells. On some occasions, the L2C cells, when stained by the anti-κ or the anti-Fab reagents, exhibited a fluorescent “cap” that occupied one pole of the cell and took up about one-sixth of the cell surface. Cells with caps generally showed no fluorescence on the rest of the cell surface. Variation in the temperature of incubation had no effect on the pattern of fluorescence observed. The L2C cells showed no fluorescence when stained with the rabbit anti-gpλ, anti-IgM, or anticolostal IgA. Control slides with a middle layer of normal rabbit serum followed by the fluoresceinated goat anti-rabbit IgG were uniformly negative. These results indicate that approximately one-third of normal lymph node lymphocytes and virtually every L2C cell has immunoglobulin on its surface.
Fig. 1. L\(^3\)C cells are stained with fluorescein-labeled anti-Fab reagent; almost all cells demonstrate staining in a speckled pattern.

**Immunofluorescent Staining by Antihistocompatibility Antiserum**

When the strain 13 antistrain 2 antiserum (diluted 1:10) was used in the indirect technique, followed by a fluoresceinated rabbit antigp\(^\tau_2\) that did not stain the cells directly, 50\%–60\% of the L\(^3\)C cells demonstrated a fluorescent cap of dramatic proportions (Fig. 2); the other 40\%–50\% of the cells had a diffuse pattern of speckled staining. Only a rare cell had both patterns simultaneously. Normal strain 2 lymph node cells demonstrated 80\%–90\% of cells with diffuse speckled staining; only a rare cell had a fluorescent cap; however, these were smaller than those observed in the L\(^3\)C cell.

**In Vitro Synthesis of Immunoglobulin**

The supernatant from the short-term culture of the L\(^3\)C cells, when studied by immunoelectrophoresis and radioimmunoauctography, revealed a line of precipitation with rabbit anti-gp\(^\tau_2\); no precipitation was seen with anti-\(\tau_1\), anti-IgM, or anticostral IgA. The radioimmunoelectrophoresis with the anti-\(\tau_2\) antibody is shown in Fig. 3. It is unlikely that the immunoglobulin secretion observed was due to contamination by the very small number of normal lymphocytes present in the peripheral blood of the leukemic guinea pig.
Fig. 2. L²C cells stained by indirect technique for presence of strain 2 histocompatibility antigens. Majority of cells show large cap at one end of cell.

*Presence of Lymphocyte Complement Receptor*

When the L²C cells were incubated with the SRBC-rabbit antibody-mouse complement complexes (EAC), 75%–80% of the cells exhibited “rosettes” with three or more RBC adhering to the surface of the cell (Fig. 4). A control population of normal strain 2 lymph node cells demonstrated 35%–40% rosette-forming cells. The crucial role of complement in the rosette formation was confirmed by the finding that the RBC-antibody complexes without added complement (EA) bound to less than 1% of the lymph node cells; the L²C cells failed to form any rosettes when incubated with EA alone.

**Table 1. Fluorescent Staining of L²C Cell and Normal Lymph Node Cells by Anti-immunoglobulin Reagents**

<table>
<thead>
<tr>
<th>Reagent</th>
<th>L²C Cell</th>
<th>Lymph Node Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-T₄ (indirect)</td>
<td>90%–100% of cells speckled</td>
<td>30%–40% of cells speckled</td>
</tr>
<tr>
<td>Rabbit anti-Fab (direct)</td>
<td>90%–100% of cells speckled</td>
<td>30%–40% of cells speckled</td>
</tr>
<tr>
<td>Rabbit anti-L chain (indirect)</td>
<td>90%–100% of cells speckled</td>
<td>30%–40% of cells speckled</td>
</tr>
<tr>
<td>Rabbit anti-T₃ (indirect)</td>
<td>Negative</td>
<td>Rare weakly staining cell</td>
</tr>
<tr>
<td>Rabbit anti-IgM (indirect)</td>
<td>Negative</td>
<td>Not done</td>
</tr>
<tr>
<td>Rabbit anti-IgA (indirect)</td>
<td>Negative</td>
<td>Not done</td>
</tr>
<tr>
<td>Normal rabbit serum (indirect)</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

*When indirect technique was used, fluoresceinated goat anti-rabbit IgG was used in second stage of procedure.*
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Fig. 3. Center well contains guinea pig serum; upper panel reveals precipitation with anti-\( \gamma_1 \) and anti-\( \gamma_2 \) reagents ("moustache" effect is due to presence of \( \gamma_2 \) in the anti-\( \gamma_1 \) reagent. This material \([\gamma_2]\) was used in soluble form to absorb rabbit serum so as to render it specific for \( \gamma_1 \).) Radioautograph in lower panel shows precipitation only with anti-\( \gamma_2 \) reagent.

additional interest in the observation that when normal strain two lymph node cells were incubated with fluoresceinated rabbit anti-gp Fab, washed with serum-free medium, and then incubated with EAC, all the cells that were positive for fluorescence demonstrated rosette formation. No cells exhibited fluorescent staining without having rosettes. Thus, those lymphoid cells that possess surface immunoglobulin, as detected by immunofluorescence, also demonstrate a complement receptor as indicated by rosette formation with EAC.

Response of \( L_2C \) Cell to Mitogens

As can be seen from Table 2, the \( L_2C \) cells failed to respond to stimulation with any of the mitogens used over a wide range of mitogen concentrations. Comparable concentrations of mitogen cause a 5–100-fold stimulation of normal strain 2 lymph node cells.
DISCUSSION

Our studies have shown that the L2C leukemia cell shares certain well-defined immunologic properties with the bone marrow derived or B population of lymphocytes. These properties include the presence of surface immunoglobulin, the secretion of immunoglobulin in vitro, the presence of the lymphocyte receptor for complement, and the failure of the L2C cell to respond to mitogens that are known to stimulate primarily thymus-derived lymphoid cells.

Evidence for the relationship of surface immunoglobulin to the B cell population comes from several sources. Lymphocytes of bursectomized chickens do not respond in culture to stimulation with antiimmunoglobulin reagents, nor do they possess surface immunoglobulin by immunofluorescence. Patients with X-linked agammaglobulinemia (Bruton type) also lack surface immunoglobulin on peripheral blood lymphocytes. Since agammaglobulinemic chickens and X-linked agammaglobulinemic humans have intact thymus systems and normal cellular immunity, these results indicate that the bursa (or its equivalent in mammals) is the source of immunoglobulin-bearing cells.

The origin of the immunoglobulin on the surface of lymphocytes has re-
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Table 2. Response of L²C Cell and Lymph Node Cell to Mitogens

<table>
<thead>
<tr>
<th></th>
<th>L²C Cell</th>
<th>Lymph Node Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Exp. 1</td>
<td>Exp. 2</td>
</tr>
<tr>
<td>PHA 0.1 T/ml</td>
<td>22,778</td>
<td>24,773</td>
</tr>
<tr>
<td>PHA 1 T/ml</td>
<td></td>
<td>26,394</td>
</tr>
<tr>
<td>PHA 5 T/ml</td>
<td>19,006</td>
<td>24,888</td>
</tr>
<tr>
<td>Con A 1 T/ml</td>
<td>22,219</td>
<td>24,425</td>
</tr>
<tr>
<td>Con A 10 T/ml</td>
<td>28,778</td>
<td>15,264</td>
</tr>
<tr>
<td>Poke 1 T/ml</td>
<td></td>
<td>21,889</td>
</tr>
<tr>
<td>Poke 10 T/ml</td>
<td></td>
<td>27,364</td>
</tr>
<tr>
<td>Poke 100 T/ml</td>
<td></td>
<td>26,080</td>
</tr>
</tbody>
</table>

Results are expressed as cpm/2 x 10⁶ cells; each value is mean of three determinations.

Recently been the topic of considerable investigation.¹⁹,²⁰ Previous studies have raised the question whether the surface immunoglobulin detected by the fluorescent antibody technique is an actual product of the cell or simply an extracellular immunoglobulin that happens to attach to the lymphocyte surface. Our studies show that the L²C cell has only ¹² immunoglobulin on its surface and secretes only ¹² immunoglobulin in vitro. It is unlikely that the specificity in both surface immunoglobulin and in immunoglobulin released into the media would exist unless both were products of the cell. Our experiments thus add support to the concept that surface immunoglobulin is a product of the cell that bears it.

The L²C cell has another property of the bone marrow-derived cell population, and this is the lymphocyte receptor for antigen-antibody-complement complexes (EAC). The properties of the lymphocytes bearing the receptor for EAC have recently been well characterized in the mouse by Bianco et al.¹⁴ These investigations have demonstrated that the population of lymphocytes that bears the membrane receptor for EAC coincides with the population that has surface immunoglobulin. The component of complement responsible for this interaction between antigen-antibody complexes and lymphocytes is C₃. The complement reactive lymphocytes (CRL) are absent from the thymus and constitute only 10%-20% of thoracic duct lymphocytes. In lymph nodes the CRL are confined to the cortical follicles (the bursa-dependent area) and are not seen in the paracortical area (the thymic-dependent area).²¹ This lymphocyte receptor for EAC can be distinguished from the monocyte receptor for EAC that requires the presence of the Mg++ ion and from the monocyte receptor for antigen-antibody complexes (EA) that does not require the presence of complement.

Of additional interest in the course of these studies was the finding of intense fluorescent staining in the form of a cap at one pole of the L²C cell when an antihistocompatibility antiserum was used. The usual pattern of speckled fluorescence was also observed on about 40% of the cells in any given preparation. When normal strain 2 lymph node cells were stained by this reagent, they revealed either ringlike or diffuse patchy patterns of stain-
ing. Cerrotini and Brunner\textsuperscript{22} have described the distribution of mouse histocompatibility antigens as small irregular areas of fluorescence separated by unstained regions. The electron microscope studies of Aoki et al.\textsuperscript{23} using the hybrid antibody technique, have shown that the distribution of mouse H-2 antigens is always discontinuous over the surface of the cell. The polar distribution of histocompatibility antigen on the surface of the L2C cell is a distinctly unusual finding.

The L2C cell fails to respond in culture to PHA and several other common mitogens. Experiments in the chicken have shown that the cells of the neonatally thymectomized animal do not respond to PHA;\textsuperscript{24} the lymphocytes of the bursectomized chicken, on the other hand, respond normally. In the human, the peripheral blood lymphocytes of patients with the DiGeorge syndrome do not respond to PHA,\textsuperscript{25} while those from patients with X-linked hypogammaglobulinemia respond normally.\textsuperscript{26} Thus, responsiveness to PHA in culture appears to be a property of the T cell population. The cells from patients with chronic lymphatic leukemia also do not respond to PHA in 3-day cultures.\textsuperscript{27} The failure of both the L2C cell and the CLL lymphocyte to respond to PHA may be indicative that both may be members of the PHA nonresponsive B cell population. However, the L2C leukemia behaves clinically as an acute leukemia, and the cells obtained from these animals may be already dividing at a maximum rate; any stimulation due to mitogens may, therefore, be difficult to observe.

The availability of a large number of pure B lymphoid cells will provide a useful tool for the study of cellular receptors of lymphoid cells, the precise manner in which immunoglobulins are attached to cell membranes, the nature of the complement receptor, and the distribution of histocompatibility antigens on cell surfaces. Furthermore, experiments from our laboratory suggest that the L2C leukemia may be an extremely useful reagent for the production of specific anti-guinea pig T cell and anti-guinea pig B cell antisera. Thus, a rabbit anti-guinea pig thymocyte antiserum has been produced that, following absorption with L2C cells, loses all specificity for bone marrow-derived cells and is highly specific for thymocytes and thymus-derived lymphocytes. Studies are also in progress to develop an anti-bone marrow-derived lymphocyte antiserum by immunizing rabbit with L2C cells and absorbing the antiserum with thymocytes.

In this paper, we have demonstrated that the L2C guinea pig leukemia cell is a member of the bone marrow-derived population of lymphocytes. Among human neoplasms, chronic lymphatic leukemia may represent an example of a B cell malignancy, since a high percentage of CLL cells have recently been shown to contain surface immunoglobulin.\textsuperscript{28} Furthermore, unlike the findings of Michlmayr and Huber,\textsuperscript{29} studies from our laboratory have shown that some CLL cells also possess the complement receptor.\textsuperscript{30} We believe that the application of the techniques described in this paper to classify other lymphoid neoplasms as to their T or B cell origin may prove rewarding.

ACKNOWLEDGMENT

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ment receptor test and Dr. Richard Asofsky for his help in performing the radioimmuno-electrophoresis. Mrs. Susan Pickeral provided technical assistance.

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