

## HUMORAL IMMUNOSTIMULATION

### V. Selection of Variant Cell Lines\*

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The mechanisms whereby cancer cells escape immune destruction are a subject of importance to the biologist and clinician alike. One conceivable mechanism is that unspecified factors *in vivo* select for variant clones with enhanced growth potential and increased resistance to the original immune response. In short-term experiments *in vitro* we have shown that antibodies exert a dual action on tumor cell growth with the expected cytotoxic effects at high antibody concentrations but stimulation of cell growth at low antibody concentrations. Limiting amounts of anti-L-cell antibody stimulate nucleoside incorporation, DNA synthesis, and cell growth in mouse L cells (1-3). Interestingly, complement (C) was found to enhance the stimulatory as well as the inhibitory effects of antibody. C activated through the third component reduced the quantity of antibody needed to produce stimulation of cell growth by as much as 1,000-fold (4). Recently we have demonstrated that this stimulation effect is reproducible in T-cell-depleted animals, thus proving that antibody *per se* can stimulate tumor growth *in vivo* (5).

In order to gain insight into the long-term effects of stimulation amounts of antibody upon cell growth and metabolism, L cells were grown *in vitro* in the presence of anti-L-cell antibody for several months. Evidence will be presented that this procedure selects for variant cells which exhibit profound differences in morphology, size, growth pattern, growth rate, susceptibility to stimulation and inhibition of cell growth by antibody, surface antigenic content and distribution, and class of antibody-binding site. A preliminary report of this work has been made (6).

#### Materials and Methods

*Parent Cell Lines.* The parent cell line was the mouse L cell (7) which was maintained at 37°C in an humidified 5% CO<sub>2</sub>-air atmosphere in monolayer cell culture in plastic T flasks with nutrient medium (NM)<sup>1</sup> composed of Eagle's minimum essential medium with glutamine plus 10% fetal calf

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<sup>1</sup> *Abbreviations used in this paper:* AL, rabbit anti-L-cell serum; ALC<sub>1</sub>, rabbit anti-LC<sub>1</sub> antiserum; CR, control rabbit serum; FCS, fetal calf serum; hNM, nutrient medium containing heat-inactivated FCS; <sup>125</sup>IUdR, <sup>125</sup>I-5-iodo-2'-deoxyuridine; L, L cells; LC<sub>1</sub>, variant clone of L; LC<sub>2</sub>, variant clone of LC<sub>1</sub>; NM, nutrient medium; PBS, 0.15 M NaCl-0.01 M phosphate, pH 7.4.

serum (FCS). The NM contained 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 50  $\mu$ g/ml kanamycin. The cell cultures were shown to be mycoplasma free by Thomas Brotherton, Department of Pediatrics, Washington University School of Medicine, St. Louis, Mo. Trypsin (1:250; Difco Laboratories, Detroit, Mich.) containing 740 benzoyl-L-arginine ethyl ester U/mg of enzyme (3) was used to subculture the cells.

*Variant Cell Lines.* The variant cell lines were selected by growing the cells in NM in the continuous presence of a 1:200 dilution of rabbit anti-L-cell antiserum (AL). Control flasks contained the same dilution of control rabbit serum. Variant cell lines were cloned from single cells. Serial 10-fold dilutions of a trypsinized cell suspension were plated into sterile 3 cm plastic Petri dishes. The Petri dishes ( $10^7$  dilutions) were examined at 4 and 24 h to insure that the resulting clones were derived from single cells. After 1 wk of culture in the Petri dishes the cells were removed and cultured in T flasks as described above. Variants of the variant cell lines were selected by growth in the continued presence of a 1:20 dilution of antiserum (cytotoxic dose). These cell lines were cloned in a similar manner.

*Antisera.* Antisera were raised in rabbits by immunization with washed cell-complete Freund's adjuvant emulsions as previously described (1). Two injections ( $2 \times 10^8$  cells each) with complete adjuvant and one injection with cells in buffer were made; the time interval between injections was 2 wk. Control rabbit serum (CR) was obtained by injection of a buffer-complete Freund's adjuvant alone. Sera were heat-inactivated at 56°C for 30 min, sterile filtered, and stored at -20°C. FCS components which may have adhered to the washed L cells have been shown not to be important immunogens for the resultant antiserum (3).

*Cell Growth Rate.* Cell lines were plated in NM either in T flasks (250 ml) or in 12 x 75 mm plastic culture tubes. At successive 24-h intervals, replicate cell monolayers or cell pellets were digested for 10 min with 0.10% trypsin, pipetted into single-cell suspensions, and counted with a hemocytometer.

*Stimulation Studies.* Cell monolayers were trypsinized for 2-5 min with 0.05% enzyme, scraped, pipetted into single-cell suspension, and plated in 1 ml of NM at a cell density of 50,000/ml. Small volumes of 0.01 M phosphate-0.15 M NaCl at pH 7.4 (PBS), CR, or anti-L-cell antiserum were added and the cells were incubated for various periods of time at 37°C.  $^{125}$ I-5-iodo-2'-deoxyuridine ( $^{125}$ IUdR, Amersham/Searle Corp., Arlington Heights, Ill., original sp act 200-2,000 mCi/mmol) at a final radioactivity concentration of 0.05  $\mu$ Ci/ml was added for 24-h time periods before harvest (1).  $^{125}$ I retained in cell pellets has been shown to be incorporated into nuclear DNA (3).

*Measurement of Cell Dimensions.* Measurements of cell diameters were made on freshly trypsinized cell suspensions with a microscope equipped with an ocular lens calibrated in 2.35  $\mu$ m at a 400 magnification. 200 cells were counted per determination. In one instance a comparison was made between the method of assessing cell size and that using a Nuclear Chicago electronic particle counter equipped with a multichannel analyzer through the courtesy of Dr. Carlton Stewart, Section of Cancer Biology, Washington University School of Medicine. There was excellent agreement between the two methods of assessing cell size.

*C-Mediated Cytotoxicity.* Cell monolayers were digested with 0.05% trypsin solution for 2 min, scraped, pipetted into single-cell suspension, and washed three times with NM containing 10% heat-inactivated FCS (hNM). Tubes containing  $2-5 \times 10^5$  cells in 1 ml of hNM were incubated at 37°C for 1 h with small amounts of PBS, CR, or antiserum in the presence and absence of small amounts of guinea pig serum obtained from Hartley animals. The cells were washed three times with hNM, plated in 1 ml of hNM in 12 x 75 mm plastic tubes at cell densities of  $0.5-1 \times 10^5$ /ml, and incubated at 37°C for 24 h with  $^{125}$ IUdR. In some experiments 20- $\mu$ l aliquots were also placed in plastic microwell grids. At the conclusion of the incubation period the cells in the tubes were assayed for residual radioactivity and those in the wells were scored for adherence and viability after fixation and staining (3,8,9). A cytotoxicity index (Table II) of 1.00 indicates no cell killing.

*Fluorescence Microscopy.* Cells were harvested with 0.05% trypsin for 2 min, washed three times in NM, suspended in 1 ml of NM containing 1% sodium azide, and incubated at 4°C for 60 minutes with PBS CR (1:50 dilution), or anti-L antiserum (1:50 dilution). After washing, the cells were incubated in NM containing 1% sodium azide at 4°C for 30 min with 0.1% vol/vol fluoresceinated goat antirabbit IgG (Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill.). Finally the cells were washed four times with PBS. One drop of FCS was added to the cell pellets; the

resulting suspension of cells was smeared into a thin layer and allowed to dry. The slides were examined under 90% buffered glycerin with a Leitz Ortholux fluorescence microscope using an episcopic attachment (E. Leitz, Inc., Rockleigh, N. J.).

**Radioiodination of IgG.** The technique of Marchalonis (10) was used to radioiodinate CR and anti-L-cell IgG fractions which had been prepared from rabbit sera by precipitation of the globulin fractions with  $(\text{NH}_4)_2\text{SO}_4$  at 40% of saturation at 0°C followed by chromatography over DEAE-cellulose columns equilibrated with 0.02 M phosphate buffer at pH 7.0 (11). Lactoperoxidase (7.5  $\mu\text{g}$ , Sigma Chemical Co., St. Louis, Mo.),  $\text{Na}^{125}\text{I}$  (0.5 mCi, Amersham/Searle Corp.), and  $\text{H}_2\text{O}_2$  (final molarity 0.005 M) were added to 1.5 mg of IgG dissolved in 250  $\mu\text{l}$  of PBS. After the reaction had proceeded 10 min at room temperature, it was stopped by the addition of 0.5 ml of 0.005 M cysteine and the product was dialyzed exhaustively at 4°C until the dialysate contained less than 2,000 cpm/ml. Approximately 1 out of 130 AL IgG and 1 out of 120 of CR IgG molecules were labeled with  $^{125}\text{I}$ , assuming one molecule of  $^{125}\text{I}$  per molecule of radioactive IgG. Univalent (Fab') fragment of AL IgG, prepared according to the methods of Nisonoff et al. (12) and Fleischman et al. (13) with minor modifications (14), was labeled with  $^{125}\text{I}$  in a similar manner. 1 of 200 Fab' molecules was thusly labeled.

**Binding of  $^{125}\text{I}$ -IgG to Cells.** Cells were harvested either by a 2 min exposure to a 0.05% trypsin solution as described above or by merely scraping nonconfluent, untrypsinized monolayers of cells and by repetitive pipetting to achieve single-cell suspensions. After washing in NM the cells were distributed and pelleted in 13 x 100 mm glass tubes by centrifugation (440 g) and aspiration. Experiments were performed on  $10^3$ - $10^7$  cells per tube but in most experiments  $1.0 \pm .5 \times 10^6$  cells per tube were used.  $^{125}\text{I}$ -IgG preparations (containing 1.6  $\mu\text{g}$  of protein and 640,000 cpm initially) were added in 10- $\mu\text{l}$  vol. The specificity of the AL antibody was demonstrated by a five- to eight-fold increase in binding to L as compared to CR and by the fact that no specific binding was seen with a human colonic cancer cell line which was not stimulated by AL. In saturation experiments nonradioactive IgG preparations (AL and CR) were added as carrier to increase the total quantity of  $^{125}\text{I}$ -IgG in the system. Calculation of total binding was based on the ability of cold IgG to inhibit  $^{125}\text{I}$ -IgG binding to cells. The cells were incubated at 4°C for 60 min before washing with 1% FCS in ice-cold PBS and centrifugation (1,500 g) four times followed by quantitative transfer to new glass tubes. Using this procedure the blank contained only 0.1% of the total counts per minute added. All binding curves were corrected for nonspecific binding of CR IgG (up to 10% in some instances). By performing cell saturation experiments it was determined that a maximum of 5.0% of the AL IgG bound to L. The calculations (Figs. 7-9) were made for experiments using untrypsinized cells. Exposure of cells to a 0.05% trypsin solution for 2 min at 37°C decreased the total number of bound antibody molecules per cell by 30% for both L and the variant clone of L ( $\text{LC}_1$ ) but did not affect the general shapes of the resultant binding curves.

**Culture for Viruses.** L,  $\text{LC}_1$ , and the variant clone of  $\text{LC}_1$  ( $\text{LC}_2$ ) cell lines were cultured for viruses by Charles Reed, Department of Pediatrics, Washington University School of Medicine. Samples of cell cultures (both living and frozen at -70°C for 30 min and thawed) were placed on primary Rhesus monkey kidney, HEP-2, LLCMK-2, and WI-38 cell lines. The cultures were fed once per week and passed (after freezing at -70°C for 30 min) into new cultures every 3 wk (6 wk for WI-38) for a total of three passages each. The cell lines were observed daily for cytopathic effects.

## Results

Growth of cells in the presence of antibody produced demonstrable changes in four out of four cell cultures and in none of the corresponding cultures grown in the presence of CR. For example, when L cells were grown as monolayers in the presence of a 1:200 dilution of AL, they gradually assumed a different morphologic appearance (Fig. 1) from the original cell line maintained in CR at the same dilution. After a four mo period the AL-treated cells could be easily distinguished from CR-treated cells (L) which appeared morphologically identical to L cells maintained without any rabbit serum. The AL-treated cells grew in aggregated clusters whereas the CR-treated cells grew in evenly spaced cell

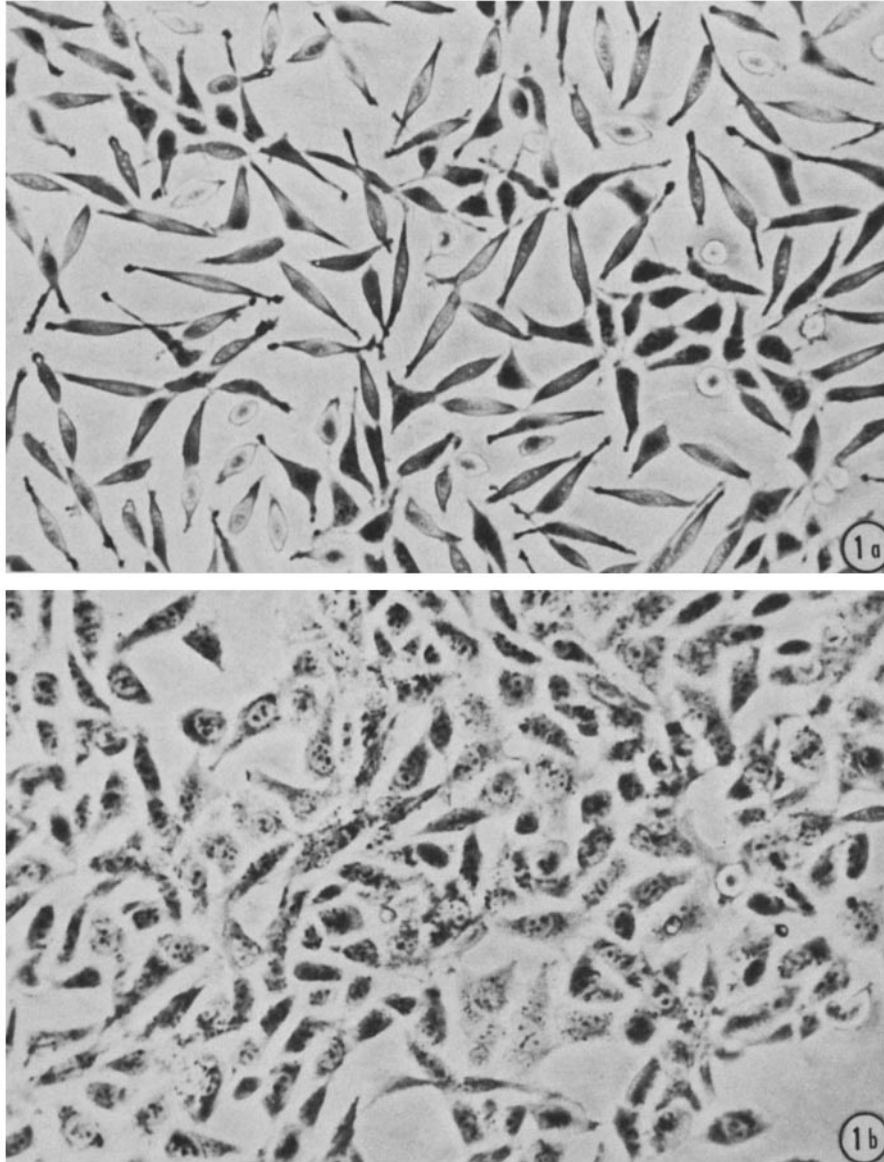


FIG. 1. Phase-contrast photomicrographs of cell monolayers. (a) L and (b) LC<sub>1</sub>.  $\times 100$ .

monolayers. The AL-treated cells quickly exhausted the NM and required more frequent subculturing. After 9 mo of growth in AL the line was cloned from a single cell (LC<sub>1</sub>) and the growth rate of L, LC<sub>1</sub>, and LC<sub>1</sub> in the presence of AL was assessed (Fig. 2). Here it can be seen that LC<sub>1</sub> grew at an enhanced rate as compared to L and that AL did not alter its growth rate. The size of freshly trypsinized (15 min) L and LC<sub>1</sub> is seen in Table I. Using the electronic particle counter the distribution of LC<sub>1</sub> cells was shifted towards larger cell volumes. Measurements by ocular microscopy confirmed these findings.

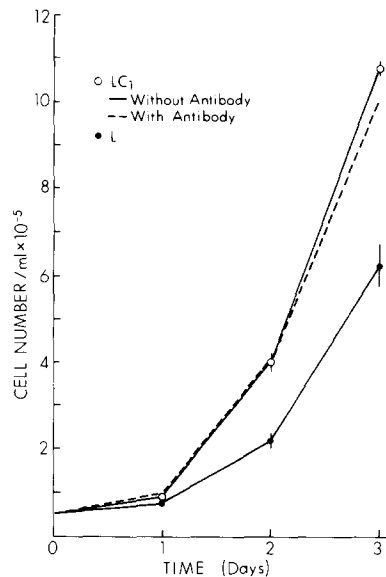


FIG. 2. Growth curves of cells in replicate T flasks initially plated at 50,000 cells/ml, harvested every 24 h, and counted. Bars represent standard errors of duplicate determinations.

TABLE I  
Dimensions of L and LC<sub>1</sub>

Method	Cell	D* ± SE	S*	Vol
		$\mu\text{m}$	$\mu\text{m}^2$	$\mu\text{m}^3$
Electronic particle counter	L	(13.1)	(539)	1,187
	LC <sub>1</sub>	(13.8)	(600)	1,384
Ocular microscopy	L	13.5 ± 0.13	(571)	(1,284)
	LC <sub>1</sub>	14.3 ± 0.16‡	(643)	(1,533)

The numbers in parentheses indicate values calculated from actual measurements. 200 cells were measured by ocular microscopy.

\* D, diameter; S, surface area.

‡  $P < 0.001$ .

Radioactive nucleoside incorporation into L and LC<sub>1</sub> in the presence and absence of AL showed that LC<sub>1</sub> no longer displayed the characteristic stimulation pattern (Fig. 3) of L. Here the stimulation ratios (counts per minute in the AL-treated cells divided by either the PBS control cells or the CR-treated cells) was greatly reduced as compared to the stimulation ratios seen with L. LC<sub>1</sub> was capable of being stimulated by its homologous rabbit antiserum (ALC<sub>1</sub>) however. In a similar experiment, for example, the stimulation ratio achieved in the 48–72 h time period with a comparable dilution of ALC<sub>1</sub> was 13.0. These findings suggested that LC<sub>1</sub> did not interact well with antiserum to the parent cell line. To further assess this possibility the susceptibility of L and LC<sub>1</sub> to C-mediated lysis in the presence of high concentrations of AL was made (Table II). Results are expressed in terms of ability to take up <sup>125</sup>IUDR and by viable cell counts of aliquots plated in microwells which were fixed and stained. Here it is obvious

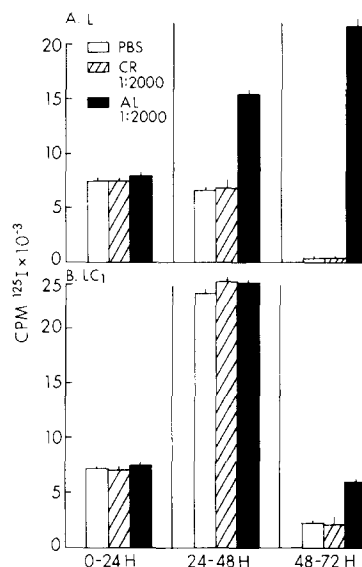


FIG. 3.  $^{125}\text{I}$ UDR uptake of cells over successive 24 h time periods. Cells were incubated in a 12 x 75 mm plastic tube in 1 ml of NM at an initial cell density of 50,000/ml with small volumes of PBS, CR, or AL (low titer).  $^{125}\text{I}$ UDR was added to replicate sets of tubes at a radioactivity concentration of 0.05  $\mu\text{Ci/ml}$ . Bars represent standard errors of triplicate determinations.

that L was killed by high concentrations of AL in the presence of excess C, whereas  $\text{LC}_1$  was susceptible to lysis only at the highest concentration of high titer AL. By either method of assessment of cell viability, either  $^{125}\text{I}$ UDR uptake or viable cell counts, the data indicated that  $\text{LC}_1$  interacted less well than L with AL. Fluorescence microscopy of L and  $\text{LC}_1$  showed that there was a more sparse and patchy distribution of bound IgG to the surface of  $\text{LC}_1$  indicating that a diminution and redistribution of original antigenic sites had taken place on the surface of the variant (Fig. 4).

The results described above suggested that growth of cells in the presence of antibody had selected for cells which were less susceptible to modulation by antibody but still could be killed if high enough concentrations of antibody and C were used. To see if further selection was possible L and  $\text{LC}_1$  were grown in the presence of a 1:20 final dilution of AL. L was immediately killed by the AL as seen in Fig. 5, whereas  $\text{LC}_1$  formed severely clumped clusters which grew slowly but at least in part survived this cytotoxic dose of AL. Growth of  $\text{LC}_1$  in a 1:20 dilution of CR had no visible effects on cell growth. After 1 mo of growth in the presence of a 1:20 dilution of AL, a new clone of cells was obtained ( $\text{LC}_2$ ) and grown in sufficient quantities for further studies.  $\text{LC}_2$  was tested for susceptibility to C-mediated lysis in the presence of high concentrations of AL and it was compared to L and  $\text{LC}_1$  (Fig. 6a). AL was more cytotoxic to  $\text{LC}_1$  than  $\text{LC}_2$  while L was completely killed. When rabbit antiserum was raised to  $\text{LC}_1$  ( $\text{ALC}_1$ ) it was possible to distinguish between the cell lines again because  $\text{ALC}_1$  produced more cytotoxicity with  $\text{LC}_1$  than with  $\text{LC}_2$ . No cytotoxicity was seen with L (Fig. 6b). Thus, it appeared that  $\text{LC}_2$  and L were different from  $\text{LC}_1$  with respect to certain

TABLE II  
Complement-Mediated Cytotoxicity with L and LC<sub>1</sub> in the Presence of AL

Condition	L Complement added (vol/vol)			LC <sub>1</sub> Complement added (vol/vol)		
	0%	5%	10%	0%	5%	10%
A. High titer AL						
PBS	1.00 (1.00)	0.98 (0.84)	0.92 (1.05)	1.00 (1.00)	0.96 (0.87)	0.94 (0.80)
CR 1:20	1.02 (1.06)	0.93 (0.91)	1.02 (1.08)	1.07 (0.94)	0.95 (0.98)	0.98 (0.78)
CR 1:50	0.99 (1.07)	1.04 (0.90)	0.91 (1.05)	1.04 (0.96)	0.89 (0.83)	0.97 (0.77)
AL 1:20	0.34* (0.11)*	0.01* (0.00)*	0.00* (0.00)*	1.04 (0.88)	0.95 (0.89)	0.79‡ (0.51)*
AL 1:50	0.70§ (0.75)	0.04* (0.00)*	0.05* (0.00)*	1.04 (0.82)	0.94 (0.81)	1.00 (0.84)
B. Low titer AL						
PBS	1.00 (1.00)	0.93 (1.12)	0.95 (0.89)	1.00 (1.00)	0.96 (0.99)	0.94 (1.05)
CR 1:5	0.83 (0.95)	0.82 (0.99)	0.97 (0.98)	0.99 (0.98)	0.95 (0.91)	0.92 (1.00)
CR 1:20	0.93 (0.86)	0.90 (1.04)	0.83 (0.93)	1.04 (1.14)	0.98 (0.82)	0.95 (0.96)
AL 1:5	0.80 (1.09)	0.73§ (0.50)‡	0.28§ (0.10)*	0.96 (1.08)	1.06 (0.88)	0.94 (0.92)
AL 1:20	0.91 (0.94)	0.80 (1.08)	0.77 (1.00)	1.01 (1.16)	0.96 (0.82)	1.00 (0.93)

Results are expressed in terms of the cytotoxicity index which is the mean measurement of the experimental divided by that of the PBS condition alone. Numbers without parentheses indicate results with <sup>125</sup>IUDR (added at a concentration of 0.05  $\mu$ Ci/ml) uptake from 0–24 h (triplicate determinations). Numbers in parentheses indicate results with cells scored for adherence and viability after 24 h incubation in microwell plates (6 or 10 determinations). Cell density in different experiments ranged from 0.7 to 1.4  $\times 10^5$ /ml. PBS values for the <sup>125</sup>IUDR assay were: 12,557  $\pm$  52 (cpm  $\pm$  SE), 10,160  $\pm$  124, 8,319  $\pm$  845, 6,408  $\pm$  95 for (A) L and LC<sub>1</sub>; (B) L and LC<sub>1</sub>, respectively. PBS values for the cell viability assay were: 266  $\pm$  13, 178  $\pm$  13, 166  $\pm$  28, 173  $\pm$  7 for (A) L and LC<sub>1</sub>; (B) L and LC<sub>1</sub>, respectively. High titer antiserum was harvested 2 mo after immunization and low titer antiserum was harvested 5 mo after immunization.

\*  $P < 0.001$ .

‡  $P < 0.05$ .

§  $P < 0.01$ .

surface membrane antigens. The decreased interaction of AL with LC<sub>1</sub> was not due to an overall loss of surface antigens since these studies with anti-LC<sub>1</sub> antibodies revealed the acquisition of new determinants not present on the parent cell line.

Interestingly, once the cell variants had been selected and isolated they retained their altered growth characteristics, even in the absence of added antibody. It is unlikely that enough residual antibody was carried over the earlier selection process to explain the continuing growth pattern since the cells had been maintained for at least 4 mo without AL and no imm noglobulin could be detected on their surface by immunofluorescence.

The binding of <sup>125</sup>I-AL IgG to cells revealed that there were fewer binding sites on LC<sub>1</sub> and LC<sub>2</sub> than L, both with respect to high affinity binding sites (<2 mg/ml of AL IgG) and to total number of molecules bound per cell at saturation (Fig. 7). LC<sub>1</sub> and LC<sub>2</sub> differed at saturation only. The binding was performed at 4°C to minimize cytolytic effects of high concentrations of antibody and to insure that the measurements reflected binding of the antibody per se and not internalization since the antibody-treated cells undergo capping and endocytosis (14). In addition this permitted a direct correlation with the results of immunofluorescence studies which were also performed at 4°C. The data are uncorrected for

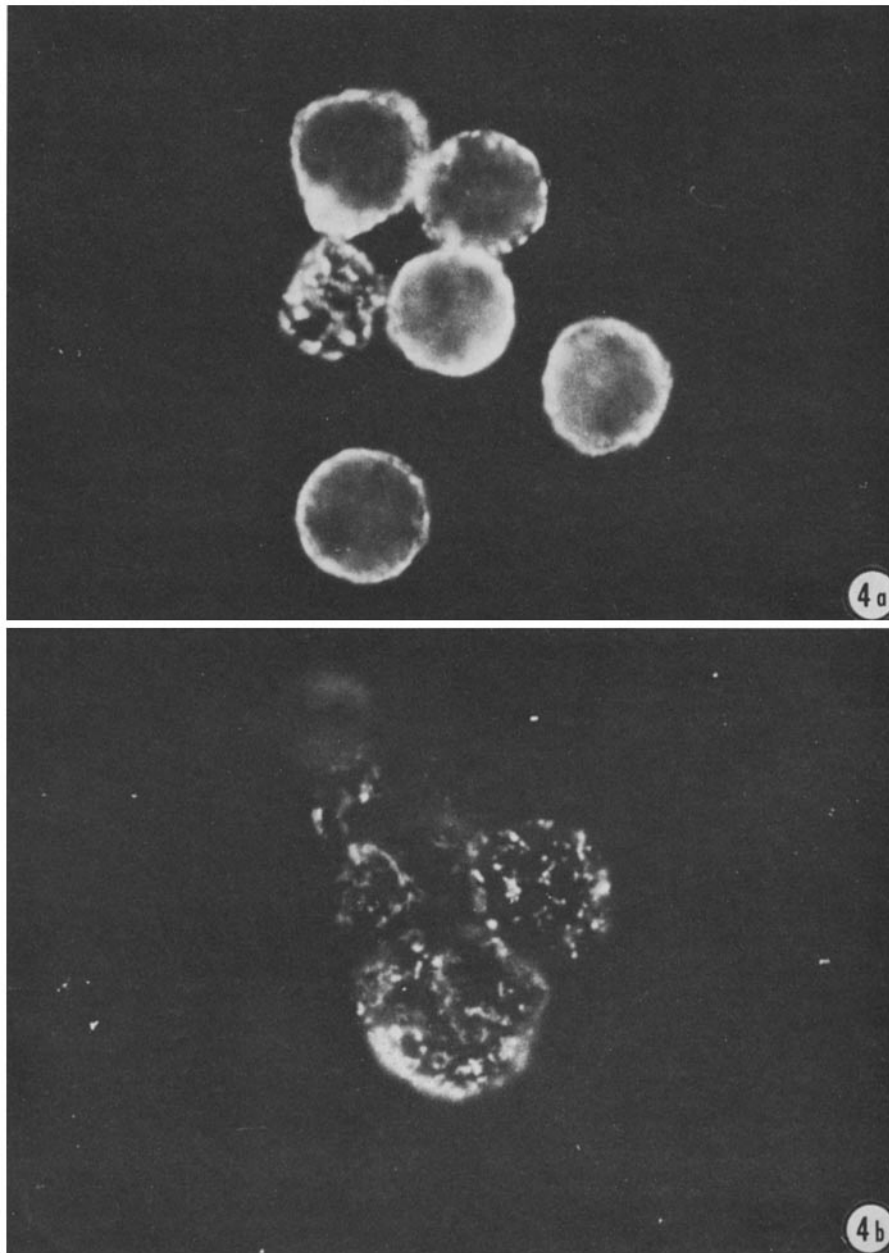


FIG. 4. Fluorescence microscopy of cells treated with AL. (a) L and (b)  $LC_1$ . Cells treated with CR did not demonstrate any fluorescence.  $\times 500$ .

surface area differences which would increase the differences between L and  $LC_1$  on a  $\mu^2$  basis.

The  $^{125}\text{I}$ -IgG-binding data were examined in greater detail by treatment of the data according to the Scatchard equation  $r/c = nK - rK$  (15), where  $r$  is moles of antibody bound per cell,  $c$  is free specific antibody molar concentration,  $n$  is the



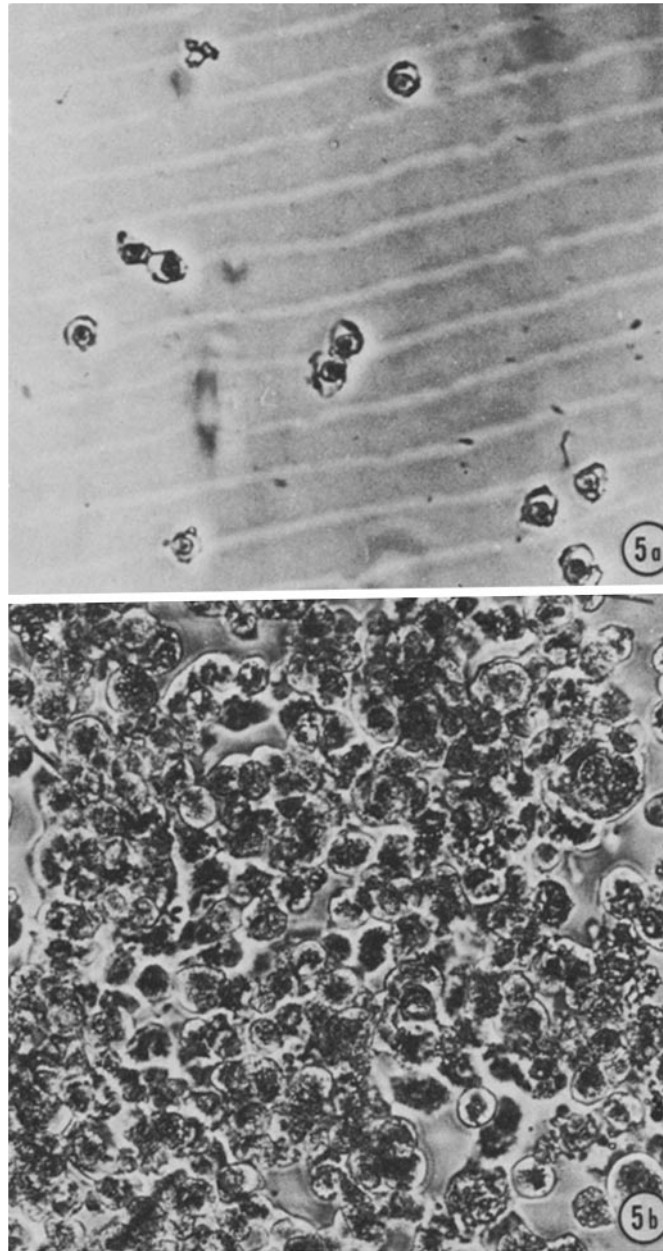


FIG. 5. Phase-contrast photomicrograph of cells. (a) L and (b)  $LC_1$ . Cells were grown for 48 h in the presence of a 1:20 dilution of high titer anti-L-cell antiserum.  $\times 100$ .

total possible number of bound antibody molecules per cell (limiting value of  $r$  at infinite  $c$ ), and  $K$  is the association constant. The resultant graphical representation of the data seen in Fig. 8, where  $r$  is the abscissa and  $r/c$  the ordinate, again demonstrates the differences between L,  $LC_1$ , and  $LC_2$ . Extrapolation of the data of  $r/c = 0$  yielded values of  $n$  of  $2.2 \times 10^7$ ,  $1.4 \times 10^7$ , and  $0.84 \times 10^7$  for L,  $LC_1$ , and

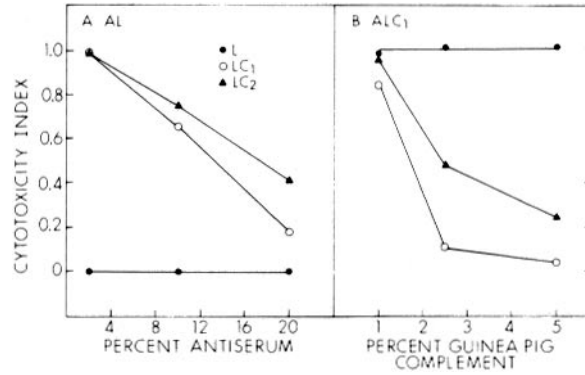


FIG. 6. C-mediated cytotoxicity with L, LC<sub>1</sub>, and LC<sub>2</sub> in the presence of high titer AL (A) and anti-LC<sub>1</sub> antiserum (ALC<sub>1</sub>) (B). Anti-LC<sub>1</sub> was harvested 2 mo after immunization. In (A) the guinea pig C was held constant at 10% vol/vol while the AL was varied; in (B) the ALC<sub>1</sub> was held constant at 1% vol/vol while the guinea pig C was varied. See legend to Table II. All differences observed between LC<sub>1</sub> and LC<sub>2</sub> are slightly significant on a statistical basis. For example, the counts per minute  $\pm$  SE for LC<sub>1</sub> at 20% AL (1:5 dilution) in (A) were  $2,910 \pm 420$  while those for LC<sub>2</sub> were  $7,230 \pm 120$ . The counts per minute  $\pm$  SE for the PBS controls were  $16,000 \pm 510$  and  $17,800 \pm 200$  for LC<sub>1</sub> and LC<sub>2</sub>, respectively.

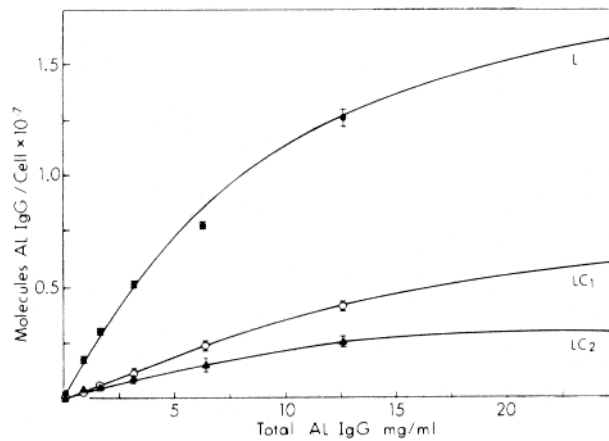


FIG. 7. Binding of <sup>125</sup>I-AL IgG to untrypsinized cell lines. Results are expressed as the mean of triplicate determinations. Bars represent standard errors.

LC<sub>2</sub>, respectively. These values can only be taken as an approximation since the extrapolations were considerable, especially for the variant cell lines. The average association constants, obtained by calculation of  $1/c$  at half-maximal saturation (e.g.,  $r = 1.92 \times 10^{-17}$  for L), were  $2.8 \times 10^5 \text{ M}^{-1}$ ,  $0.9 \times 10^5 \text{ M}^{-1}$ , and  $1.0 \times 10^5 \text{ M}^{-1}$  for L, LC<sub>1</sub> and LC<sub>2</sub>, respectively. Very similar values can be obtained by taking the negative slopes of the straight part of the curves as the  $K$ 's. It is quite apparent from Fig. 8 that considerable heterogeneity of antibody binding exists since the plots are no longer linear as  $r$  approaches zero. For all cell lines there were at least two classes of binding sites (high and low) based upon antibody affinity. Extrapolation of the initial slopes to  $r$  (e.g., to  $0.78 \times$

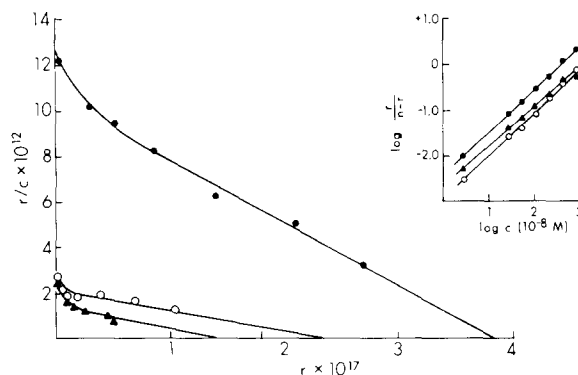


FIG. 8. Scatchard plot of binding of  $^{125}\text{I}$ -AL IgG to cell lines. Data and symbols are taken from Fig. 7. Insert: Sips plot of data.

$10^{-17}$  for L) yields  $K$ 's of  $2.6 \times 10^6 \text{ M}^{-1}$ ,  $1.3 \times 10^6 \text{ M}^{-1}$ , and  $1.4 \times 10^6 \text{ M}^{-1}$  for L,  $\text{LC}_1$  and  $\text{LC}_2$ , respectively, which are approximately 10-fold higher than the low affinity  $K$ 's. The approximate number of high affinity sites was  $0.47 \times 10^7$  or 21% of the total binding sites for L. Corresponding values for  $\text{LC}_1$  and  $\text{LC}_2$  were  $0.18 \times 10^7$  (13%) and  $0.12 \times 10^7$  (14%), respectively. Although these calculations cannot be considered precise they do indicate that the parent line and the variant lines differ both qualitatively and quantitatively with respect to different classes of antibody-binding sites. It is very probable that there are populations of binding sites with much greater antibody affinity ( $>10^7 \text{ M}^{-1}$ ) as well.

As an additional examination of the  $^{125}\text{I}$ -IgG-binding data the Sips equation (16)  $\log r/n - r = a \log K + a \log c$  was utilized, where the symbols are the same as those used previously and  $a$  (heterogeneity constant) represents the slope of the line (insert, Fig. 8). The values of  $n$  were assumed to be those obtained by use of the Scatchard equation. By taking the reciprocal of the free antibody concentration when one-half of all of the binding sites on the cell are occupied by antibody, i.e. when  $\log r/n - r = 0$ , the  $K$ 's obtained are  $2.8 \times 10^5 \text{ M}^{-1}$ ,  $0.83 \times 10^5 \text{ M}^{-1}$ , and  $1.0 \times 10^5 \text{ M}^{-1}$  for L,  $\text{LC}_1$ , and  $\text{LC}_2$ , respectively. As can be seen from the plotted data the  $K$ 's for the variant lines could be obtained only by extrapolation since 50% of the antibody-binding sites on the cells were not actually titrated in the experiment, assuming the values of  $n$  are correct. Nevertheless, the average association constants are in substantial agreement with those obtained with the use of the Scatchard equation. As could be expected the values of  $a$  were all less than 1.0 (0.9, 0.9, 0.85 for L,  $\text{LC}_1$ , and  $\text{LC}_2$ , respectively) indicating heterogeneous binding between cells and antibody.

In general there was good correlation between fluorescence microscopy, cytotoxicity, and binding data using L and  $\text{LC}_1$  in that by every parameter measured there appeared to be less original antigen upon the surface of  $\text{LC}_1$ . Studies with  $^{125}\text{I}$ -AL Fab' fragment showed that although fluorescence microscopy revealed a different distribution of antibody-binding sites of  $\text{LC}_1$ , redistribution alone was not the sole reason for lack of reactivity with AL. Even though a full saturation curve was not measured, it is clear from Fig. 9 that there is an absolute decrease of antibody-binding sites on  $\text{LC}_1$  as assessed by the binding of univalent anti-

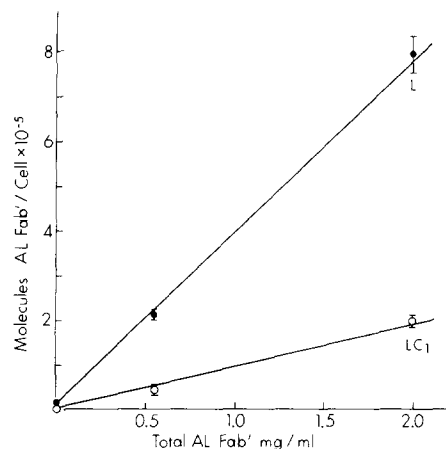


FIG. 9. Binding of  $^{125}\text{I}$ -AL Fab' to cell lines. See legend to Fig. 7.

body. All results of attempts to grow viruses from the variant and parent cell lines were negative.

### Discussion

When selective pressures are exerted by exogenous agents on suitable mammalian cell populations ultimately highly resistant cells are produced. The process is similar in concept and practice to the selection of bacterial mutants although the mechanism by which the diversity is generated is unknown and in neoplastic cells there is a surprisingly high frequency of successful selection even in the absence of mutagenic agents. In addition to a number of well studied examples in which cells no longer susceptible to cytotoxic drugs or radiation have been obtained, selection on the basis of toxic events occurring at the cell surface appears possible. Thus Chinese hamster ovary cells no longer sensitive to the cytotoxic effects of concanavalin A (17), phytohemagglutinin (17), or ricin (18) have been isolated and antisera to human cell lines have been used to select for hybrid cell lines deficient in a lethal antigen (19).

Judging from the results of recent studies of antibody effects on tumor cell growth in vitro, as discussed in the introduction, at least two types of cell selection unfavorable to the host involving changes in the antigenic content of the cell surface could theoretically occur: (a) In the presence of limiting amounts of antibody or a low surface antigen density an increase in tumor-specific antigen content might render the cells more susceptible to stimulation by antibody. (b) In the presence of high concentrations of cytotoxic antibody a diminution in surface antigen content might render the cells less susceptible to the cytotoxic effects of antibody, with or without an increase in the stimulatory effects of antibody. These effects were particularly marked with mouse L cells and mouse anti-L-cell antibody. The present study was carried out in an attempt to determine whether one or both of these mechanisms could be shown to operate in the L-cell system in vitro and this has been clearly demonstrated. Clones obtained from cells grown in the presence of antibody for an extended period have been shown to be markedly altered in their growth properties and suscepti-

bility to stimulatory and inhibitory effects of antibody. This was not the result of arbitrary cell selection during cloning since altered clones of cells were repeatedly obtained from cultures containing AL but not from cultures containing CR.

Interestingly, thus far the variants selected are less susceptible both to the stimulatory and inhibitory effects of antibody on cell growth. These changes appear to be associated with changes in the surface antigen content of the cells. Not only do the variant cells bind fewer antibody molecules, but the affinity distribution and density of their surface antigen molecules appears to be substantially altered. The change in binding and susceptibility to antibody-mediated effects on cell growth is not due to an overall elimination or masking of antigenic groups on the cell surface or metabolic alterations in these cells making them more resistant to immune modulation. Antibodies to variant cells bind to and kill variant cells more effectively than the original cell line. Thus, there has been a qualitative alteration in antigenic structures on the cell surface.

The demonstration that variant cells which bind anti-L antibody less effectively than the original L-cell line are less susceptible to cytotoxic action might have been predicted since a number of studies have shown a direct relationship between antigen content and susceptibility to C mediated cytotoxicity (e.g. 20, 21). The diminished ability of anti-L antibodies to stimulate cell growth in the variant cells would suggest that stimulation as well as inhibition is favored by a high surface antigen content and that quite possibly the same antigenic determinants are involved in two responses. The failure to dissociate the inhibitory and stimulatory effects of antibody is not too surprising since both phenomena are markedly potentiated by C and would thereby be favored under conditions in which fixation of C is maximized. However, it should be noted that thus far only one antiserum dilution has been used during the original selection phase and it is possible that different results will be obtained when lower antibody concentrations are studied.

The basis for the cell selection leading to the development of antibody-resistant cell lines is not presently known but several possible mechanisms can be considered: (a) It is possible that small numbers of cells present in the original culture were selectively stimulated by antibody and eventually overgrew the other cells in the culture. However, if this were the mechanism it is hard to understand why the variant cells eventually isolated were not longer stimulated by antibody. (b) It is possible that most of the cells with a high antigen content were killed, allowing cells unaffected by the antibody to grow more rapidly. If this were the mechanism one might have expected the variant cells to overgrow the culture in any case, since they grow considerably more rapidly than the original cells. However, it is possible that under the usual culture conditions nonvariant cells deplete the medium of nutrients of critical importance in the growth of variant cells, keeping the number of variant cells in the culture to a minimum. (c) Perhaps the most interesting possibility is an initial modulating effect of the antibody on cell metabolism which is eventually translated into permanent alterations in cellular behavior. Thus, it is possible that the antibody is analogous to a tumor-promoting agent which acts over a period of hours to days on a suitable target cell leading eventually to transforma-

tion. Under such a scheme the loss of surface antigens originally present on these cells would be due to cell dedifferentiation per se rather than a selection of pre-existing cells with a low antigen content.

Whatever the mechanism of selection it is apparent that once the variant cells have been isolated they can be maintained by serial passage in vitro for at least several months without altering their growth or immunological characteristics. To our knowledge this is the first successful attempt to immunologically select for cells with a sustained alteration in their growth and antigenic properties in an entirely in vitro system.

Old et al. (21) demonstrated a loss in TL antigens from cells exposed briefly to TL antibody in vitro but the TL content of the cells was fully restored after several cell divisions. Working in the mouse Moloney lymphoma cells, Fenyö et al. (22) were unable to select for stable immunoresistant cell lines by exposing cells to antibody and C in vitro despite use of 20 cycles of exposure in vitro followed by passage in vivo in a nonimmunized host. Stable immunoresistant cells were obtained by serial passage in vivo in animals preimmunized with irradiated cells. Aside from being smaller in size than the original cell line the variant lymphoma cells were similar to the variant L cells obtained in this study. The variant cells had decreased amounts of the original antigen on their membranes, and were resistant to lysis by antibody. Using SV-40-induced hamster tumors Deichman and Kluchareva (23) also selected in vivo for immunologically resistant tumor, but it was not clear whether the cells were changed in immunogenicity or immunosensitivity.

While the frequency and ultimate effect of selection analogous to that observed in vitro in this study on immune resistance in vivo is not presently known, this is an important subject for future study. Our in vitro evidence suggests the possibility that one long-term effect might be to select for a rapidly growing variant tumor cell which can no longer be controlled even by high concentrations of antitumor antibody. While the variant cells appear to contain new or altered antigenic determinants which might stimulate an immune response of their own, such a response might be delayed or rendered less effective because of the phenomenon of original antigenic sin. Judging from the studies of Fenyö et al. (22) with the Moloney lymphoma described above, it seems likely that the selection process we have observed in vitro is also operative in vivo. However, since only one immunologically resistant lymphoma subline was obtained (it subsequently gave rise to a subline of its own on further serial passage), it is conceivable that the appearance of the resistant line was fortuitous and documentation is needed. In addition, the results with this tumor are complicated by the presence of live virus and it is possible that antibody effects may be occurring at least in part through interference with the viral infectivity or, as mentioned above, the relationship of long-term antibody-induced cell selection to short-term stimulatory effects of antibody on cell growth presently known.

It has been recently shown that limiting amounts of highly purified and heterospecific antibodies directed to surface membrane antigens of several transformed cell lines stimulated nucleoside uptake, DNA synthesis, and cell growth (1-3). In addition, concomitant activation of C acted synergistically with

antibodies to augment cyto stimulation (4). As evidence that this same phenomenon can occur in vivo we have shown that animals devoid of cellular immunity demonstrated enhanced L-cell tumor growth with passively administered anti-L-cell antibody (5). Changes in L-cell growth were seen within 2-3 wk after tumor transplantation and antibody administration. While these in vivo observations appear to fit quite well with older work dealing with classical experimental models for immunologic enhancement which suggested that morphologic changes do indeed take place in the enhanced tumors (24-26), the long-term effects of antibody L-cell growth in vivo have not yet been studied and the relative role of immunoselection as opposed to immunostimulation of cell growth requires clarification. In this connection, it will be of considerable interest to obtain cells from the enhanced tumors and compare their immunological reactivity and growth patterns with those of cells maintained or selected in vitro.

### Summary

A permanent L-cell variant cell line ( $LC_1$ ) was isolated by the growth of the parent L-cell line (L) in the presence of a cyto stimulatory dose (1:200) of rabbit anti-L-cell antiserum (AL) for 9 mo.  $LC_1$  differed from L in many aspects: (a) it was larger ( $1,533 \text{ mm}^3$  vs.  $1,284 \text{ mm}^3$ ), (b) it grew faster (1.5- to 2-fold), (c) it grew in aggregated fashion, (d) its growth was no longer stimulated by AL, (e) it was almost completely resistant to high concentrations of AL in the presence of complement (C), (f) its original membrane antigens (immunogenic for AL) were redistributed in sparse and patchy clumps as noted by fluorescence microscopy, (g) it contained about 65% of the total original  $^{125}\text{I}$ -AL membrane-binding sites ( $1.4 \times 10^7/\text{cell}$  vs.  $2.2 \times 10^7/\text{cell}$ ), (h) its AL-binding sites displayed a lower average affinity constant ( $K = 0.9 \times 10^5 \text{ M}^{-1}$  vs.  $2.8 \times 10^5 \text{ M}^{-1}$ ), (i) it contained a smaller proportion of high affinity ( $K > 10^6 \text{ M}^{-1}$ ) binding sites (13% vs. 21%), and (j)  $LC_1$  was fully immunogenic in that it was readily killed by homologous antiserum ( $ALC_1$ ) and C, whereas L was not similarly affected by  $ALC_1$  indicating that  $LC_1$  contained new membrane antigens not present on L.

Another variant ( $LC_2$ ) was produced by growth of  $LC_1$  in a 10-fold higher dose (1:20) of AL (cytotoxic for L) for 1 mo.  $LC_2$  was even more resistant to AL in the presence of C, contained  $0.84 \times 10^7$  AL-binding sites/cell with an average affinity constant of  $1 \times 10^5 \text{ M}^{-1}$  (unchanged from  $LC_1$ ), and was less susceptible than  $LC_1$  to lysis in the presence of  $ALC_1$  and C.

These findings confirm and extend our previous in vitro and in vivo observations dealing with the direct stimulation effects of antibody on tumor cell metabolism and suggest that immunostimulation may be a mechanism of tumor escape from immune control in vivo possibly by immunoselection and antigenic modulation as proposed by other investigators.

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*Note Added in Proof.* We wish to include mention of the work of Hyman (27), Hyman and Stallings (28), and Hyman et al. (29), who describe isolation of stable variants of

murine leukemia-lymphoma cell lines and present evidence to suggest that the variant cells (insensitive to antibody) selected in their systems pre-existed in the parental cell population and that exposure to antiserum did not induce the variants.

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