Independently arising macrophage mutants dissociate growth factor-regulated survival and proliferation

(cell survival/tumor progression/colony-stimulating factor 1/growth factor/autocrine)

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ABSTRACT Analysis of a simian virus 40-immortalized colony-stimulating factor 1 (CSF-1)-dependent macrophage cell line (BAC1.2F5) and independently arising autonomous mutants derived from it (aut4A, aut4A.1, aut2A, and aut2A.1) revealed distinct phenotypes. The parental line, BAC1.2F5, is dependent on CSF-1 for survival and growth. Of the mutants derived from BAC1.2F5, aut4A has lost the requirement of CSF-1 for survival; aut4A.1 (derived from aut4A) and aut2A grow in the absence of growth factor but proliferate more rapidly in its presence, and aut2A.1 (derived from aut2A) produces CSF-1 and proliferates as rapidly in the presence as in the absence of exogenous CSF-1. The separation of the CSF-1 requirement for survival and proliferation observed in aut4A is also observed in a temperature-sensitive (ts) mutant *tsgro1*. At the nonpermissive temperature, *tsgro1* cell proliferation is arrested, but the cells survive provided CSF-1 is present. The four cellular phenotypes observed—immortalization, loss of growth factor requirement for survival, loss of growth factor requirement for proliferation, and loss of growth factor-stimulated proliferation—indicate a divergence of the pathways of growth factor-regulated survival and proliferation and may represent phenotypes occurring at intermediate stages in tumor-cell progression.

The introduction of foreign genes into cells either by retroviral transformation or DNA transfection has led to the characterization of oncogenes and the discovery that two or more oncogenes are required for cellular transformation of cultured primary cells (1–4). Similarly, in studies with transgenic mice, it has been reported that the expression of two oncogenes in conjunction with at least one other genetic alteration is necessary for the development of breast (5) or prostatic (6) cancer. Furthermore, this conclusion has been supported by experiments in which the introduction of a growth factor gene into cells expressing its cognate receptor has not resulted in malignant transformation without further selection, presumably associated with other genetic alterations (7, 8). Thus, the experimental evidence, based on several different approaches, indicates that loss of growth control involves several distinct steps. Such steps could include loss of control of cell survival, proliferation, or differentiation.

Another approach to studying loss of cell-growth control is to analyze variants of growth factor- or steroid hormone-dependent cell lines (9, 10). We have used the simian virus 40-immortalized, colony-stimulating factor 1 (CSF-1; also known as macrophage colony-stimulating factor, M-CSF)-dependent mouse macrophage cell line, BAC1.2F5 (11), to isolate mutants with less-stringent growth factor requirements. In this paper, we describe the properties of five new

mutants, each possessing a different type of growth factor responsiveness.

MATERIALS AND METHODS

Cell Culture. Cells were cultured in α minimal essential medium supplemented with 15% fetal calf serum and 1.32 nM pure mouse L cell CSF-1 (growth medium) as described (11). Cell viability was assessed by culturing cells in growth medium in tissue culture dishes (Falcon) for 21 days prior to fixing, staining, and counting colonies. In some instances, viability was assessed by trypan blue exclusion. There was a close correspondence between trypan blue permeability and loss of viability assessed by plating efficiency. Plating efficiencies in semisolid (0.3% agar) medium were measured by culturing cells in growth medium with and without CSF-1 for 3 weeks. For preparation of conditioned medium, cells were seeded in triplicate at 2.5×10^4 cells per ml in 35-mm tissue culture dishes and incubated for 7 days without changing the medium. Conditioned media were harvested, centrifuged (800 g for 10 min at 4°C), and filtered through a 0.4- μ m filter.

Isolation of Mutants. For the isolation of clones aut2A and aut4A, subconfluent 100-mm tissue culture dishes of individual BAC1.2F5 clones were exposed to 300 μ g of ethyl methanesulfonate (EMS) (Eastman) per ml for 18 hr in growth medium; the monolayers were washed and cultured for 3 days in growth medium to allow expression of mutations prior to selection for the autonomous phenotypes. Selection was performed by culturing the cells for 3 weeks in growth medium without CSF-1 and replacing the medium every 3–4 days. Individual clones were picked from separate plates, grown up in growth medium, and recloned through two cycles of limiting dilutions in 96-well tissue culture plates (Linbro/Becton Dickinson) in growth medium with (aut4A) or without (aut2A) CSF-1. aut2A.1 was cloned through two cycles of limiting dilution from a mass culture of aut2A cells that had been continuously cultured in the absence of CSF-1. aut4A.1 was isolated from aut4A cultures by placing aut4A cells at subconfluent densities (5×10^6 per 100-mm plate) and culturing them in the absence of CSF-1 for 3 weeks. Clones were picked and recloned through two cycles of limiting dilution. For the isolation of temperature-sensitive (ts) mutants, BAC1.2F5 cells were initially cultured for 10 days at 39.5°C to kill existing ts mutants. Cells were then exposed to EMS at 100 μ g/ml for 40 hr at 34°C, the medium was replaced, and the cultures were incubated for 6 days at 34°C

Abbreviations: CSF-1, colony-stimulating factor 1; ts, temperature sensitive; EMS, ethyl methanesulfonate.

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for expression of mutations. Selection was then performed by culturing 4×10^6 mutagenized cells in 4×100 mm Petri dishes (Falcon) in growth medium lacking ribo- and deoxyribonucleosides at 39°C for 24 hr, exposing to [^3H]thymidine (40–60 Ci/mmol; 2 $\mu\text{Ci}/\text{ml}$, Amersham; 1 Ci = 37 GBq) in the same medium for 24 hr at 39°C , washing, and culturing in CSF-1-containing medium with nucleosides for 6 days. This selection cycle was repeated, and the surviving cells were twice-cloned at 34°C by plating in CSF-1-containing medium in 96-well tissue culture plates at 0.25 cell per well. One of three clones isolated (tsgr01) exhibited a ts phenotype and was expanded for further study.

Other Methods. Purification of L cell CSF-1, its radioiodination with retention of biological activity, the binding of ^{125}I -labeled CSF-1 (^{125}I -CSF-1) to cells, and the CSF-1 RIA were performed as described (12). Northern analysis of RNA prepared from cells was carried out as described (13).

RESULTS

Mutants That Dissociate Growth Factor-Regulated Survival from Proliferation. Earlier studies of CSF-1-induced survival and growth indicated that survival could be differentially stimulated by maintaining primary macrophages in low concentrations of the growth factor (14). One of the clones (aut4A) appearing in the selection for CSF-1 independence could not be recloned in the absence of CSF-1. Therefore, it was recloned in medium containing CSF-1, and its growth characteristics were analyzed. In the presence of CSF-1, aut4A cells behaved like BAC1.2F5 cells (Fig. 1 A and B). However, in the absence of CSF-1, in contrast to BAC1.2F5 cells, aut4A cells remained viable without a significant in-

FIG. 1. Growth and survival curves for BAC1.2F5 and its aut4A mutants. (A and B) Growth curves of BAC1.2F5 (circles), aut4A (triangles), and aut4A.1 (squares) in the presence (filled symbols) and absence (open symbols) of CSF-1. Each growth curve was repeated at least twice. (C and D) Relative plating efficiencies in the presence of CSF-1 of cells cultured in the absence of CSF-1 for the times indicated (abscissa). Symbols are as in A and B. Each point represents the mean $\pm$ SD of triplicate determinations.

crease in cell number (Fig. 1 A–D). aut4A cells could be maintained as subconfluent cultures in the absence of CSF-1, with medium changes but without subculture, for at least 2 months. aut4A cell viability was not maintained by production of extracellular CSF-1, since CSF-1 could not be detected in the medium from cultures of aut4A cells [the limit of detection is 5 pM; 220 pM CSF-1 is required for complete survival without proliferation (11, 12, 14)] (Table 1).

Dissociation of CSF-1-stimulated survival and proliferation was also suggested by the analysis of a mutant with a ts phenotype for CSF-1-induced proliferation (tsgr01). In the presence of CSF-1, tsgr01 cells cultured at 34°C grew more slowly than BAC1.2F5 cells (doubling times 30 and 26 hr, respectively) (Fig. 2B). However, compared with BAC1.2F5 (Fig. 2A), tsgr01 cell numbers ceased to increase within 48 hr after elevation of the temperature to 39°C (Fig. 2B). At this temperature, the cells remained 100% viable by Trypan blue exclusion for at least 4 days. This effect of the nonpermissive temperature was reversible on shift down to 34°C (Fig. 2B). However, while CSF-1-stimulated tsgr01 cell growth was inhibited at 39°C , the survival of the cells was still dependent on CSF-1, as shown by the decrease in cell number on its removal (Fig. 2C). Thus, the results with aut4A and tsgr01 are consistent with the existence of separate pathways of CSF-1-induced survival and proliferation.

Mutants Whose Proliferation Is Enhanced by CSF-1. Two independently selected mutants, aut4A.1 and aut2A, exhibited a phenotype in which proliferation was observed in the absence of CSF-1 and was enhanced by the addition of CSF-1 to a rate approaching that of BAC1.2F5 cells. aut4A.1 cells were selected as a proliferating clone from unmutagenized aut4A cells cultured in the absence of CSF-1 as described in *Materials and Methods*. The frequency of clones such as aut4A.1 in aut4A cultures maintained in the presence of CSF-1 for 4 weeks was approximately 4×10^{-7} . In contrast to aut4A cells, aut4A.1 cells proliferated in the absence of CSF-1 with a doubling time of 47 hr but proliferated more rapidly (doubling time, 39 hr) in its presence (Fig. 1B and Table 1). Interestingly, low concentrations of CSF-1 were found in the medium from aut4A.1 cells cultured in the absence of CSF-1 (Table 1). aut2A cells were obtained in a single-step selection from EMS-mutagenized BAC1.2F5 cells. The doubling time of aut2A cells was 46 hr in the absence of CSF-1 and 32 hr in its presence, and the saturation density attained in the absence of CSF-1 was significantly

Table 1. Properties of BAC1.2F5 and its mutant clones

Clone	Doubling time, * hr		CM CSF-1,† pM	CSF-1R complement,‡ %	Agar cloning, % plating efficiency	
	Without CSF-1	With CSF-1			Without CSF-1	With CSF-1
BAC1.2F5	∞	28	0	100	0.3	23.8
aut4A	C	36	0	71 $\pm$ 3	0.4	19.4
aut4A.1	47	39	20	43 $\pm$ 3	3.6	19.2
aut2A	46	32	0	88 $\pm$ 8	34.2	50.6
aut2A.1	32	30	332	13 $\pm$ 4	10.4	27.4
tsgr01 (34°C)	∞	30	ND	126 $\pm$ 10	ND	ND
tsgr01 (39°C)	∞	C	ND	135 $\pm$ 11	ND	ND

C, constant cell number (no doubling); ND, not determined.

*Minor variations in cell doubling times were observed depending on the batch of fetal calf serum used. All experiments were internally controlled by including BAC1.2F5 cells.

†Cultured medium (CM) was collected from at least three different cultures and assayed in duplicate.

‡Results of two or three independent experiments assayed in triplicate $\pm$ the range or SD. Results are presented as a percentage of the number of CSF-1 receptors (CSF-1R) expressed on the parental BAC1.2F5 cell line (120,000 receptors per cell; ref. 11).

FIG. 2. Comparison of CSF-1-stimulated survival and proliferation of parental BAC1.2F5 cells and the tsgro1 mutant at 34°C and 39°C. (A) Growth curves for BAC1.2F5 at 34°C (circles) and 39°C (squares) in the presence of CSF-1 (●, ■, and ▲) and following its removal at day 5 (○ and □). (B) Growth curves of tsgro1 cells at 34°C (●) and after the following temperature shifts: δ 34–39°C (■) and δ 34–39°C/ δ 39–34°C (▲). (C) Effect of removal of CSF-1 on the survival of tsgro1 cells at 39°C: growth curve for tsgro1 cells at 34°C (circles), growth curve for tsgro1 cells subjected to a 34–39°C temperature shift at day 2 (squares), and growth curves after removal of CSF-1 at day 5 from half of the cultures (open symbols). Each point represents the mean $\pm$ SEM of the mean of triplicate determinations. Experiments shown in A, B, and C were repeated at least twice.

less than in its presence (Fig. 3 and Table 1). The CSF-1 independent proliferation of aut2A cells was not due to the release of CSF-1 because the culture medium was devoid of this growth factor (Table 1). Mutants of this type occur in the absence of mutagenesis with a frequency of 3×10^{-6} and represent the most common mutant phenotype isolated in single-step selections. The frequency of cells of this phenotype that arise independently is enhanced 10- to 100-fold by mutagenesis with EMS (J.W.P., C. Boocock, C.J.M., P.D.S., and E.R.S., unpublished data).

Mutants That Are Independent of CSF-1 for Proliferation. Upon continuous culture of aut2A cells in the absence of CSF-1, it was observed that the population doubled significantly faster. Sequential sampling of the culture and RIA of the conditioned medium indicated that the CSF-1 concentration was increasing with passage number. When growth curves were performed with cells that had been cultured for 3 months in the absence of CSF-1, there was no difference in their doubling time in its presence or absence. Therefore, three clones were isolated as described in *Material and Methods*. These clones behaved similarly, and one of them (aut2A.1) was characterized further. In contrast to aut2A,

aut2A.1 had a faster doubling time in the absence of CSF-1 (32 hr compared with 46 hr), which was not significantly altered when the cells were cultured in the presence of CSF-1 (Fig. 3 and Table 1), and the saturation densities in the presence and absence of CSF-1 were similar. In contrast to the results with aut2A, the medium from aut2A.1 cells grown in the absence of exogenous CSF-1 contained CSF-1 (332 pM; Table 1). Culture of aut2A.1 cells in the absence of exogenous CSF-1 but with a neutralizing goat anti-mouse CSF-1 antiserum (12) had no significant effect on the doubling time under conditions in which the CSF-1-induced proliferation of BAC1.2F5 cells was blocked (Table 2). Furthermore, the growth rate of aut2A.1 cells in the absence of CSF-1 was unaffected by daily medium changes, and the plating efficiency in the absence of CSF-1 was independent of input cell number. Similarly, CSF-1 had no effect on their plating efficiency or colony size at low cell density. Thus, the increased growth rate of aut2A.1 cells does not appear to be due to autocrine stimulation by released CSF-1. Overgrowth of the cultures by cells with the phenotypes of aut2A.1 could be prevented by continuously culturing aut2A cells under nonselective conditions in the presence of CSF-1.

CSF-1 Receptor Expression. The expression of cell surface CSF-1 receptors in the mutant clones was examined by 125 I-CSF-1 binding to upregulated cells at 4°C (Table 1). Receptor expression by aut4A and aut2A cells was only slightly lower than expression by BAC1.2F5 cells (Table 1). However, aut4A.1 and aut2A.1 cells expressed significantly lower numbers of cell surface receptors, possibly because of their down-regulation by endogenously produced CSF-1.

Table 2. Effect of anti-CSF-1 (α CSF-1) antibody on the proliferation of BAC1.2F5 and its mutants

Clone	Doubling time, hr			
	With CSF-1		Without CSF-1	
	Without α CSF-1	With α CSF-1	Without α CSF-1	With α CSF-1
BAC1.2F5	38	∞	∞	ND
aut2A.1	33	ND	30	35
aut4A.1	39	ND	47	48

The anti-CSF-1 antibody was 0.5% (final concentration) goat anti-mouse CSF-1 antiserum (12); 0.5% anti-mouse CSF-1 antiserum had no effect on BAC1.2F5 cell proliferation induced by human CSF-1 under these conditions (data not shown). ND, not determined.

FIG. 3. Growth curves for BAC1.2F5 and its aut2A mutants. BAC1.2F5 (●), aut2A (▲ and △), and aut2A.1 (■ and □) were cultured in the presence (filled symbols) or absence (open symbols) of CSF-1. Points represent the mean $\pm$ SD of at least four determinations from two independent growth curves.

Immunoprecipitated receptors from BAC1.2F5 and these four cell lines migrated with an apparent molecular mass of ≈ 165 kDa on NaDodSO₄/PAGE (P.D.S., J.W.P., and E.R.S., unpublished data). Interestingly, *tsgro1* had significantly increased receptor numbers at both the permissive (126%) and nonpermissive temperatures (135%).

Cell morphology. BAC1.2F5 cells in the absence of CSF-1 are mostly rounded with fine retraction fibers and lack intracellular vesicles (Fig. 4*a*). In the presence of CSF-1, the cells display a range of morphologies from very flattened cells to rounded ones. They are significantly more spread than in the absence of CSF-1 (15). Many cells are highly spread with ruffled lamellae and contain vesicles of various types clustered around the nucleus (Fig. 4*b*). In the absence of CSF-1, aut2A cells are smaller and even more rounded than BAC1.2F5 cells (Fig. 4*c*) and have short stubby processes. The cells are somewhat more spread in the presence of CSF-1 but do not show intracellular vesicles or membrane ruffling or display the characteristic BAC1.2F5 flattened morphology (Fig. 4*d*). aut2A.1 cells are even more rounded than aut2A cells (Fig. 4*e*), do not respond morphologically to exogenous CSF-1, and are indistinguishable in the presence or absence of CSF-1 (Fig. 4*f*). In contrast, aut4A cells, even in the absence of CSF-1, display the characteristic morphology of BAC1.2F5 cells in the presence of CSF-1. They are flattened cells with ruffled lamellae and centrally situated vesicles (Fig. 4*g*). CSF-1 results in cells being even more spread with many central small phase-dark ($0.5\ \mu\text{m}$) and larger phase-lucent vesicles (Fig. 4*h*). aut4A.1 cells have a more aberrant morphology than aut4A cells, with many cells having short radial processes (Fig. 4*i* and *j*). Nevertheless, in the absence of CSF-1, these cells are significantly more spread than BAC1.2F5 cells, containing centrally located phase-dark and phase-lucent vesicles and ruffled lamella (Fig. 4*i*). In the presence of CSF-1, aut4A.1 cells have a similar morphology to those grown in its absence (Fig. 4*j*).

Anchorage-independent growth. Anchorage-independent growth has been directly correlated with tumorigenicity in fibroblasts (16). Macrophage colony formation by primary

cells in semisolid medium is dependent on CSF-1 (17). When BAC1.2F5 cells were cultured in agar in the absence of CSF-1, 0.3% of the cells formed colonies. In the presence of CSF-1, their plating efficiency was enhanced by ≈ 80 -fold (Table 1). aut4A cells behaved similarly to BAC1.2F5 cells, but aut4A.1 cells possessed a 10-fold higher plating efficiency in the absence of CSF-1 than BAC1.2F5. In contrast to BAC1.2F5 and aut4A cells, aut2A cells in the absence of CSF-1 possessed a high plating efficiency (34.2%), which was only slightly increased upon culture in CSF-1. However, in the presence of CSF-1, aut2A colonies were larger, as expected from their faster rate of proliferation in CSF-1. Interestingly, the plating efficiency of aut2A.1 cells in the absence of CSF-1 (10.4%) was lower than the plating efficiency of aut2A cells, although an increase to levels approaching those of aut2A cells was observed when aut2A.1 cells were cultured with CSF-1. There was no difference in colony size between aut2A.1 cells plated with CSF-1 and those plated without growth factor or aut2A plated with CSF-1. The pattern of plating efficiencies and relative colony sizes for BAC1.2F5 and all of these mutants was similar when they were cultured on tissue culture plastic, with the exception of aut2A.1, whose plating efficiency was not enhanced by CSF-1.

RNA (Northern) analyses. Since two of the aut mutants examined (aut4A.1 and aut2A.1; Table 1) produced extracellular CSF-1, RNA was analyzed by Northern blotting with a CSF-1 cDNA probe for the presence of the CSF-1 mRNA (Fig. 5). Compared with the control mouse L cell (lane 7) pregnant mouse uterus (lane 1) RNAs, which respectively expressed high levels of the 4.6-kb and of the 2.3- and 4.6-kb CSF-1 mRNAs, very low levels of expression were seen with RNA from BAC1.2F5 and the four aut mutants. Interestingly, aut2A.1 cells, which produced the highest levels of CSF-1, possessed CSF-1 mRNA at concentrations that were reproducibly indistinguishable from those of aut2A, which does not produce extracellular CSF-1. It should be noted, however, that the amount of CSF-1 produced by aut2A.1 cells is at least 10 times lower than the amount produced by mouse L cells.

FIG. 4. Phase-contrast micrographs of BAC1.2F5 cells and its mutants. BAC1.2F5 (*a* and *b*), aut2A (*c* and *d*), aut2A.1 (*e* and *f*), aut4A (*g* and *h*), and aut4A.1 (*i* and *j*) were cultured for 48 hr in the absence (*a*, *c*, *e*, *g*, and *i*) or presence (*b*, *d*, *f*, *h*, and *j*) of CSF-1.

DISCUSSION

A somatic cell genetic approach was taken to isolate autonomous mutants from the CSF-1-dependent macrophage cell line, BAC1.2F5. Immortalization of macrophages and analysis of these mutants showed there were three distinct phenotypes between primary macrophages and macrophages that exhibit full growth-factor autonomy. These phenotypes are represented by (i) BAC1.2F5 cells that were immortalized by transfection of origin-defective simian virus 40 DNA (18) but still require CSF-1 for both survival and proliferation (11); (ii) aut4A cells in which the effect of CSF-1 on survival and proliferation was dissociated; (iii) aut4A.1 and aut2A cells, which exhibit CSF-1-independent cell proliferation that is enhanced by CSF-1; and (iv) aut2A.1 cells that produce CSF-1 but no longer require the growth factor for maximal proliferation. The evidence that each of these steps in the path to growth-factor autonomy occurs as a result of independent mutations is their low frequency of spontaneous occurrence (10^{-6} – 10^{-7}), the increased frequency (10^{-4} – 10^{-5}) of variants following EMS mutagenesis, and the stability of the phenotypes in the absence of selection. These progressively more independent phenotypes may result from mutations in different genes or additional mutations in the same gene giving additive function (or loss of function). Given the diverse phenotypes and the complexity of the growth factor response, it is highly likely that the mutations are in multiple genes. In this respect, it is interesting that the single-step selection mutants aut2A and aut4A and 15 of the 17 auton-

FIG. 5. Northern blot analysis of BAC1.2F5 and mutant RNA with a CSF-1 cDNA probe. Lanes: 1, control RNA from a day 14 pregnant mouse uterus; 2, BAC1-2F5; 3, aut2A; 4, aut2A.1; 5, aut4A; 6, aut4A.1; 7, control RNA from mouse L cells. Ethidium bromide staining demonstrated equivalent concentration of 28S and 18S rRNA in each lane after transfer. Sizes are shown in kb.

omous mutants derived by single-step selection after EMS mutagenesis failed to produce detectable growth factor (P.D.S., J.W.P., and E.R.S., unpublished data), suggesting that these mutations reside in intracellular components of the signal transduction pathway. In contrast, the vast majority of spontaneously arising autonomous mutants of the factor-dependent myeloid cell line D35 produced growth factor as a consequence of retroviral insertional activation of myeloid growth factor genes (19).

The genetic dissociation between CSF-1-induced survival and proliferation observed in aut4A was also observed in tsgro1. In this mutant the proliferative response to CSF-1 was inhibited by a shift to 39°C, but the cells still required CSF-1 for survival. This indicates that inhibition of the activity of a protein required for proliferation does not result in loss of growth factor-regulated viability. The isolation of these two independent mutants provides evidence for a genetic separation of the regulation and/or the process of proliferation and viability. Previous evidence for the dissociation of these two growth factor-regulated processes was derived from experiments in which low concentrations of CSF-1 were sufficient to stimulate survival and maximally inhibit protein degradation, whereas higher concentrations were required for the stimulation of proliferation (14).

It has been clearly shown that steroid hormones can regulate cell survival in tissues subject to cyclical endocrine regulation (20). Furthermore, there is increasing evidence that the regulation of cell proliferation and survival by hormones is mediated by local growth factor expression (21). A mutation that results in the cell's failure to exhibit hormone-regulated cell death would result in inappropriate enrichment of this population, which could contribute, by subsequent hormone-regulated proliferation, to the development of a tumor. aut4A cells possess properties of such a population. Interestingly, upon further selection, aut4A cells gave rise, at a frequency of 4×10^{-7} , to cells represented by aut4A.1, which proliferated in the absence of growth factor. aut4A.1 cells exhibited a similar phenotype to aut2A cells, in that CSF-1 increased their proliferative rate. Further selection of aut2A cells for CSF-1 autonomy led to the predominance of cells with the phenotype of aut2A.1, whose proliferation rate could not be increased by addition of CSF-1. Since aut2A.1 cells produce CSF-1, they may be exhibiting autocrine regulation by this growth factor. While experiments using neutralizing anti-CSF-1 antisera failed to inhibit the growth of aut2A.1 cells, indicating that exogenously produced CSF-1 was not responsible for their proliferation, these data do not preclude a mechanism of autocrine stimulation involving intracellular receptors as has been shown in

the case of platelet-derived growth factor (22, 23). Thus, the *in vitro* behavior of the aut4 and aut2 series mimics the behavior of certain tumors *in vivo*, which show initial hormone dependence and subsequent progression to hormone independence and metastases (24). The relevance of these studies to human neoplastic disease has been emphasized by the studies of Kacinski *et al.* (25, 26), in which ovarian and endometrial tumors have been shown to coexpress CSF-1 and its receptor, and an elevated circulating CSF-1 concentration is a marker for disease in patients bearing these tumors.

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