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In vitro culture of primary plasmacytomas requires stromal cell feeder layers

(adhesion/neoplasia/plasma cells)

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ABSTRACT **Attempts to grow primary murine plasmacytomas *in vitro* have, to date, been largely unsuccessful. In this study, we demonstrate that long-term *in vitro* growth of primary plasmacytomas is accomplished by using feeder layers composed of stromal cells from the initial site of plasmacytomagenesis. The early neoplastic lines established in this manner are dependent on physical contact with the stromal layer, which is mediated in part by CD44, for growth and survival. The stromal cells provide at least two stimuli for the plasma cells, one being interleukin 6 and the second, of unknown nature, resulting from direct physical interaction that cannot be replaced by soluble factors. These plasma cell lines have been passaged for as long as 20 months yet still maintain characteristics associated with primary plasmacytomas as they will grow *in vivo* only in pristane-primed animals, indicating a continued dependence on the pristane-induced microenvironment characteristic of early-stage tumors. The ability to grow primary plasmacytomas in culture and maintain their "primary" properties provides a model system for detailed analysis of early events in plasma cell tumor progression involving neoplastic cells completely dependent on physical contact with a stromal feeder layer for survival and expansion.**

Plasmacytomas are induced in BALB/cAnPt mice by the i.p. introduction of plastic materials (1), mineral oils (2–4), or alkanes such as pristane (2,6,10,14-tetramethylpentadecane) (5). Such treatment results in the formation of a chronic granulomatous tissue on the peritoneal surfaces (2, 6, 7) that is the site of the developing plasmacytoma. Treatment with antiinflammatory drugs, such as hydrocortisone and indomethacin, inhibits plasmacytoma development (7, 8), suggesting a critical role for granulomatous tissue in early stages of plasmacytoma growth.

One of the hallmarks of primary plasmacytomas is that ascitic tumor cells are rarely successfully transplanted when introduced into the peritoneal cavities of normal, syngeneic mice (9) but grow in mice that have previously received 0.5 ml of pristane i.p. However, the dependence of primary plasmacytomas on the oil-induced microenvironment is usually lost after several transplant generations, indicating that microenvironmental and growth requirements of early developing plasmacytomas differ from those of long-term transplanted tumors. Consistent with this view has been the inability to grow primary plasmacytomas *in vitro*, whereas, in contrast, many serially transplanted plasmacytomas have been adapted to tissue culture. In this paper, we describe the development of a system that maintains the *in vivo* dependence of plasmacytomas on the oil-induced microenvironment, thus providing an opportunity to study growth requirements and early events of granuloma-dependent tumors.

MATERIALS AND METHODS

Mice and Plasmacytoma Induction. BALB/cAnPt mice derived from a conventionally reared colony maintained at Hazleton Laboratories (Rockville, MD), under National Cancer Institute Contract N01-CB-21075, were injected i.p. with pristane (Aldrich) using standard procedures (5). Tumor-bearing mice were diagnosed by the presence of at least 10 hyperchromatic tumor cells on Wright/Giemsa-stained smears of peritoneal ascitic fluid.

***In Vitro* Growth of Plasmacytoma Cells.** The mesentery from tumor-bearing mice was placed in a dish containing RPMI 1640 medium with 5 μg of fungizone per ml and 50 μg of gentamycin per ml (GIBCO). The mesenteric lymph nodes were discarded and the remaining tissue was placed in a new dish, chopped into small pieces, and placed at 37°C for 1 hr in a 50-ml tube containing 20 ml of Hanks' saline (calcium and magnesium free), 150 units of collagenase per ml (GIBCO), 0.1% trypsin (ICN), and 2% heat-inactivated chicken serum (GIBCO). The tube was then gently mixed for 5 min, after which the cells in suspension (minus large pieces) were collected, washed twice, and resuspended in tissue culture medium (RPMI 1640 containing 10% fetal calf serum, 2 mM glutamine, 0.1 mM nonessential amino acids, and 50 μg of gentamycin per ml). The remaining tissue fragments were redigested with enzyme solution. The dissociated cells were pooled, resuspended in tissue culture medium, and seeded at 2.5×10^6 per 100-mm dish. Established cultures were passed by removing plasma cells from the surface of the feeder layer, digesting the feeder layer with the above enzyme mixture for 45 min at 37°C, and replating at a ratio of 5–10 plasma cells to 1 stromal cell.

Basement membranes. Basement membrane used in the primary cultures was extracted from the extracellular matrix produced by the Engelbreth-Holm–Swarm tumor (10). The major components of the extracted material include laminin, type IV collagen, heparan sulfate proteoglycan, entactin, and nidogen.

Microchamber cultures. Microchamber primary cultures were prepared in 24-well plates by inoculating plasma cells, isolated by centrifugation on Ficoll density gradients (Pharmacia) and depleted of adherent cells on tissue culture dishes for 1 hr at 37°C, in the upper part of a chamber, which was

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Abbreviations: mAb, monoclonal antibody; IL-6, interleukin 6; r, recombinant; FITC, fluorescein isothiocyanate.

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separated by a permeable membrane (0.22 μm) (Millicell, Millipore) from the floor of the well previously inoculated with stromal cells (20,000 cells per well).

Assays and Cell Characterization. *Plasmacytoma adherence.* Adherence assays were performed as described by Miyake *et al.* (11) using ^{51}Cr-labeled plasma cells. BM2 hybridoma and BMS2 bone marrow stromal cell lines were kindly provided by Paul Kincade (Oklahoma Medical Research Foundation) and the NIH 3T3 cell line was provided by J. H. Pierce (National Cancer Institute). Monoclonal antibody (mAb)-producing cell lines YN1/1.7.4 (anti-ICAM-1/CD54), FD441.8 (anti-LFA-1/CD11a), R1-2 (anti-VLA 4), and M1/70 (anti-Mac 1/CD11b) were obtained from the American Type Culture Collection. Antibody was purified from each cell line using a protein A-Sepharose column.

Interleukin 6 (IL-6) assays. IL-6 assays were performed using the B9 hybridoma line as described (12).

Proliferation assays. Plasma cell proliferation was measured in a standard [^{3}H]thymidine assay (13). Briefly, 2.5×10^3 plasma cells per well were seeded in 96-well plates in the presence of recombinant IL-6 (rIL-6) or 5×10^3 irradiated (4000 rad; 1 rad = 0.01 Gy) stromal cells in 200 μl of medium. One hundred microliters of fresh medium was exchanged on day 3, and cultures were pulsed on day 6 with 1 μCi of [^{3}H]thymidine (1 Ci = 37 GBq) followed by collection on filters 18 hr later. Parallel cultures of irradiated stromal cells were plated alone and assayed for IL-6 activity using the B9 indicator line.

Immunoglobulin assays. Supernatants from *in vitro* cultured plasmacytoma cells were assayed for the presence of immunoglobulin using a Pandex machine (Baxter Healthcare, Deerfield, IL) (14).

Fluorescence-activated cell sorter analyses. The cell surface phenotype of *in vitro* lines was determined on a FACScan (Becton Dickinson). Viable cells were electronically gated by exclusion of propidium iodide. Cells were stained with a panel of fluorescein isothiocyanate (FITC)-conjugated mAbs specific for the following cell surface antigens: CD45 (clone 30-F11) (15), FcγRII (clone 2.4G2) (16), CD45(B220) (clone RA3-6B2) (17), ThB (clone 53-9.2) (15), Thy-1.2 (Becton Dickinson), Mac-1 (clone M1/70) (18), MEL-14 and VLA-4 (Pharmingen, San Diego), and CD44 (clone KM201) (11).

Southern analyses. Southern blots were obtained using standard procedures as described by Hilbert and Cancro (19).

Electron microscopy. Transmission and scanning electron microscopy studies were performed as described (20).

RESULTS

Establishment of *in Vitro* Cultures and Characterization of Plasma and Stromal Cells. Eight primary plasmacytomas were obtained from the peritoneal cavities of BALB/cAnPt mice previously injected with pristane. A high cell density was found to be necessary for growth, so that, for primary cultures, 10^6 cells were seeded into 60-mm plates or 2.5×10^6 cells into 100-mm plates. Six of the eight plasmacytomas were successfully expanded *in vitro* together with autologous stromal cells. One primary culture was lost to contamination and the other failed to grow, most likely due to a low number of cells initially recovered. An *in vitro* culture of a primary plasmacytoma after 1 month is shown in Fig. 1*A* with clusters of plasma cells seen adhering to the top of, and within, the stromal cell feeder layer. Established cultures of plasma cells and stromal feeder cells could be expanded for at least 20 months. After several passages the stromal cells begin to outgrow the plasma cells and it becomes more convenient to passage weekly on irradiated feeders at a ratio of 1:2–5 (plasma:stromal cell).

Transmission electron microscopy (data not shown) of cultures containing stromal cells alone revealed three morphologically distinct cell types: (*i*) mesothelial cells forming multilayers with characteristic long microvilli on the surface that are tightly attached to the plastic by adhesion plaques, (*ii*) endothelial cells forming monolayers with little or no extracellular matrix, (*iii*) fibroblastic reticular cells producing a thick basement membrane composed of amorphous fibrillar material. Transmission electron micrographs of plasma cells growing *in vitro* in association with these stromal cells reveal that the plasma cells are readily distinguished by an abundant rough endoplasmic reticulum and intracisternal alpha particles (Fig. 1*B*) and are restricted to either the microvillous

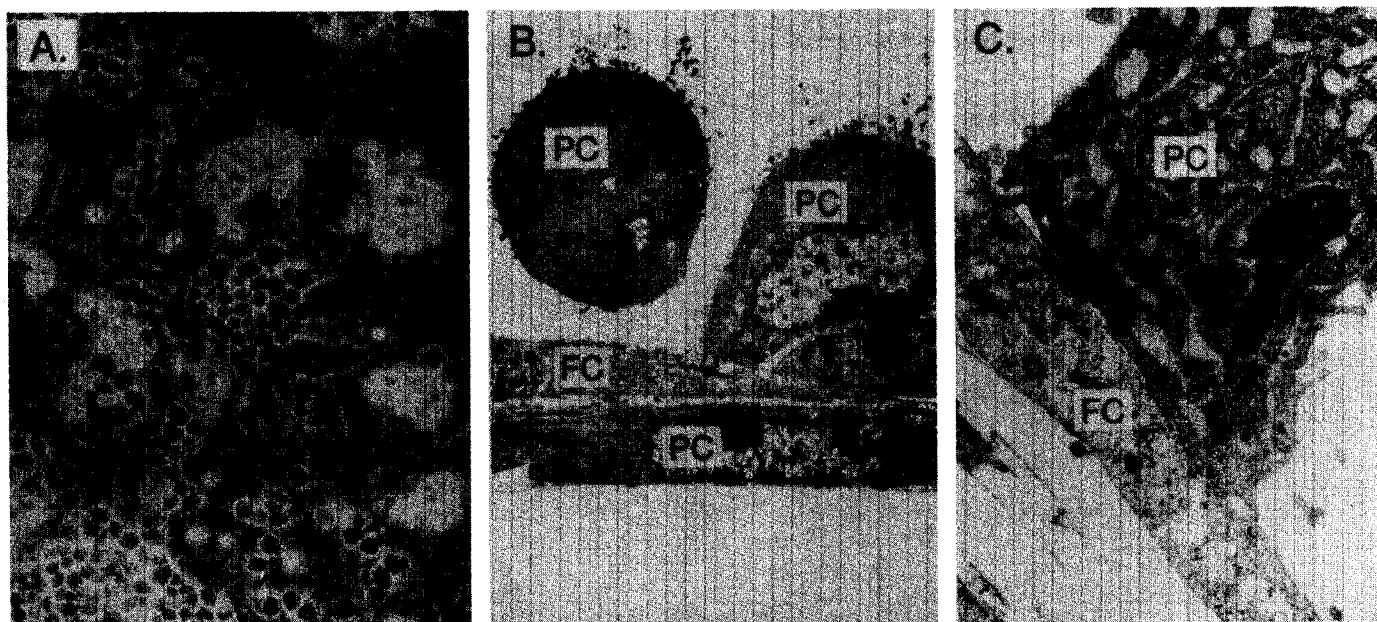

FIG. 1. (*A*) Phase-contrast micrograph showing a primary plasmacytoma growing on a confluent stromal cell layer after 1 month of *in vitro* culture: plasma cells are localized on the top (bright cells) as well as within or beneath the feeder layer (dark cells). (×60.) (*B*) Transmission electron micrograph showing plasma cell growth *in vitro* on the stromal feeder layer. PC, plasma cells; FC, fibroblastic cells. The arrow indicates a typical intracisternal alpha particle. Plasma cells are present on the top and between the stromal cell layer. (×3375.) (*C*) Junctional activity with cytoskeleton reorganization is present between plasma cells and stroma (arrows). (×4500.)

Table 1. Immunoglobulin secretion and cell surface phenotype of *in vitro* growing primary plasmacytomas

Plasma cell line	Immunoglobulin isotype†	Phenotype*								
		CD45	CD45 (B220)	ThB	Thy-1.2	Mac-1	CD44	MEL-14	VLA-4	FcγRII
5-25	IgAk	++	–	++++	–	–	+++	–	+	++
17-82	IgAk	+	–	++	–	–	+	–	±	+
6-26	k only	+	–	++++	–	–	++++	–	+	+
8-36	IgMk	+	–	ND	–	–	ND	ND	ND	+

ND, not determined.
*Cell surface phenotype of plasmacytomas was determined using direct FITC conjugates of the indicated antibodies.
†Supernatants were screened for immunoglobulin using particle concentration immunofluorescence.

surface of the mesothelial cells or the intercellular space between the mesothelial and fibroblastic reticular cells. Under higher magnification, contact between plasma cells and stroma results in cytoskeleton reorganization and deposition of dense material between the two membranes (Fig. 1*C*). Characterization of four of the plasma cell lines yielded the phenotypes presented in Table 1. To determine if the *in vitro* cell lines accurately reflect the original *in vivo* tumor, Southern blot analysis of the immunoglobulin variable heavy-chain loci was performed (Fig. 2). In each case, the rearrangements of these loci were identical in the *in vivo* tumor and the corresponding cell lines. The germ-line band in the tumor preparations represents contaminating normal cells.

Growth Requirements. As described above, the successful *in vitro* growth of primary plasmacytomas was completely dependent upon coculture on a stromal cell feeder layer. This interdependence was further indicated by attempts to grow purified plasma cells from primary explants in medium alone, in medium containing rIL-6, in medium containing rIL-6 and artificial basement membranes, in stromal cell conditioned medium, or in diffusion chambers that physically separate plasma cells from stromal cells but allow passage of soluble, stromal cell elaborated factors. None of these conditions supported plasmacytoma growth.

Analyses of the same plasma cell lines, 5-25, 17-82, and 6-26, after 8, 4, and 6 *in vitro* passages (Fig. 3), respectively, showed slight differences in growth characteristics compared to primary explants. Although none of the lines proliferated when plated alone, 5-25 and 6-26 showed significant incorporation of [^{3}H]thymidine in high concentrations of rIL-6 whereas 17-82 did not. The IL-6 levels for the stromal cell lines in parallel cultures are as follows: BMS2, 2,639 B9 units/ml; 5-25S, 11,690 B9 units/ml; 6-26S, 731 B9 units/ml; 17-82S, 322 B9 units/ml; and 3T3, 4 B9 units/ml. Furthermore, each plasma cell line proliferated well on heterologous feeders as well as the bone marrow stromal cell line BMS2 but not on 3T3 cells, which make the lowest amount of IL-6. Growth on 3T3 can be enhanced to nearly the same levels as autologous feeders by the addition of exogenous IL-6 (data not shown). Correspondingly, the growth of plasma cells on

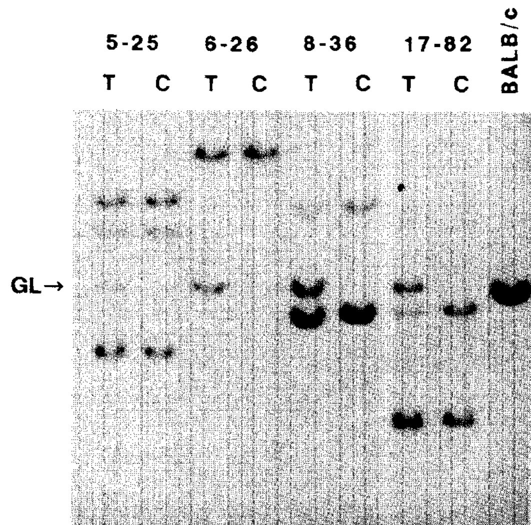

FIG. 2. Southern blot analyses of immunoglobulin rearrangements: Ten micrograms per lane of *Eco*RI-digested high molecular weight DNA from either the *in vivo* tumor (T) or the resulting *in vitro* cell line (C) was electrophoresed and hybridized with an immunoglobulin joining heavy-chain probe that detects all rearrangements. GL, germ-line band.

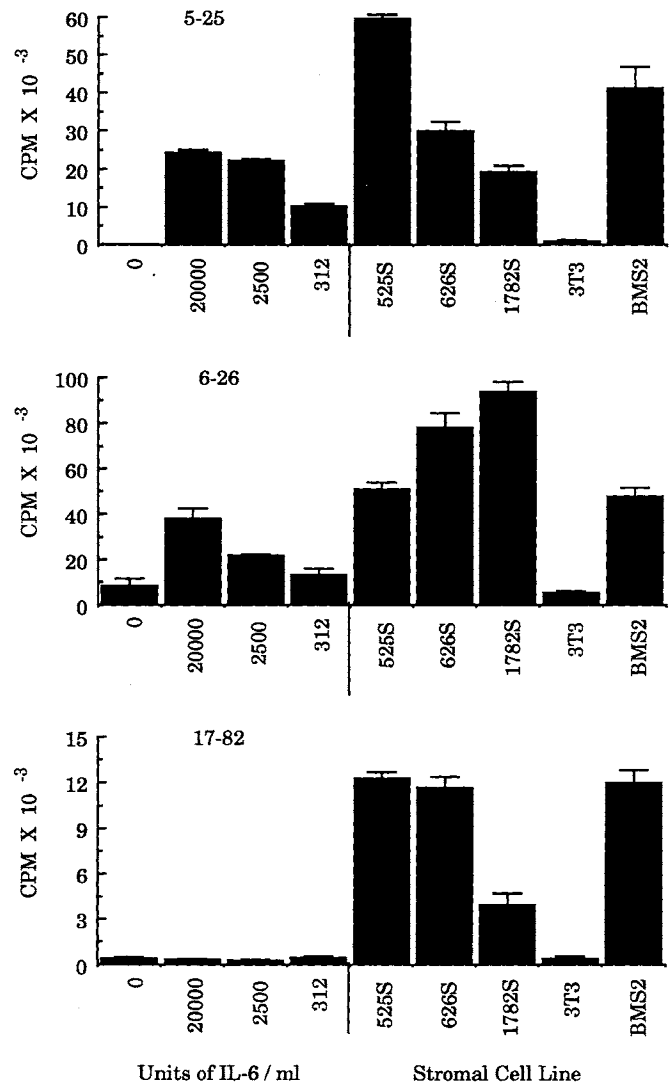

FIG. 3. Proliferation of plasma cells 5-25, 6-26, and 17-82 in the presence of rIL-6 (expressed as B9 units) or stromal cells 5-25S, 6-26S, 17-82S, 3T3, and BMS2. IL-6 values are given in B9 units. BMS2 is a bone marrow-derived stromal cell line described by Miyake *et al.* (11). Data are presented as mean ± SE for quadruplicate cultures.

Table 2. Oil-induced microenvironment is necessary for the *in vivo* growth of cultured primary plasmacytomas

Plasma cell line	Time in culture		Incidence of tumor growth	
	Passage	Months	BALB/c	BALB/c + pristane
5-25				
Exp. 1	p15	9	0/5	5/5
Exp. 2	p18	11	0/3	3/3
Exp. 3	p20	20	0/4	3/4
17-82	p9	5	0/4	4/4
6-26	p10	6	0/4	3/4
8-36	p5	3	0/4	4/4

autologous feeders can be inhibited by >85% in the presence of a polyvalent rabbit antiserum to purified, native mouse IL-6. It should also be noted that after 6 months of *in vitro* expansion, IL-6 dependent, stromal cell-independent sublines have been established from four plasmacytomas by gradual depletion of the stromal cells in the presence of 10,000 B9 units/ml of rIL-6, suggesting a possible progression from stromal cell dependence to independence.

To test whether the stromal cell-dependent plasma cell lines maintained characteristics of primary tumors and remained dependent on the pristane-induced microenvironment, 10^6 cells from four of the lines were injected i.p. into untreated mice and mice that received 0.5 ml of pristane i.p. 3 days prior to plasma cell transfer. None of the untreated mice developed tumors within 20 weeks, whereas all of the *in vitro* lines produced tumors in pristane-primed mice within 4–6 weeks (Table 2).

Plasma Cell Adhesion. The mechanism of adhesion of plasma cells to stromal elements has been approached following the observation of Miyake *et al.* (11) that hybridomas bind to bone marrow stromal cells via interaction of the cell surface molecule CD44 and its ligand, hyaluronate. Three plasma cell lines were assayed for adhesion to stromal lines via hyaluronate (Fig. 4). In all cases, binding to hyaluronate in the absence of feeder cells was completely eliminated by prior treatment of plasma cells with hyaluronidase. Hyaluronidase reduced binding of plasma cell lines 5-25, 17-82, and 6-26 to autologous feeders by 52%, 80%, and 52%, respectively. Interestingly, all lines adhere well to BMS2, but inhibition by hyaluronidase is a maximum of 72% with 17-82 and far less with the other two lines. It should be noted that

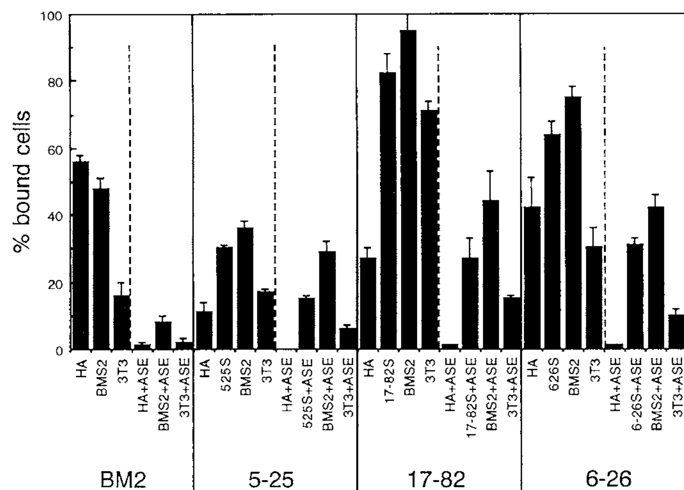

FIG. 4. Effect of hyaluronidase treatment on adhesion of plasma cells to hyaluronic acid (HA) or stromal cells BMS2, 5-25S, 17-82S, 6-26S, and 3T3. Treatment with testicular hyaluronidase (ASE) (5000 units/ml) was performed at 37°C for 1 hr. Data are presented as mean ± SE for quadruplicate wells. The bone marrow-derived cell line BMS2 and the hybridoma BM2 were included as positive controls.

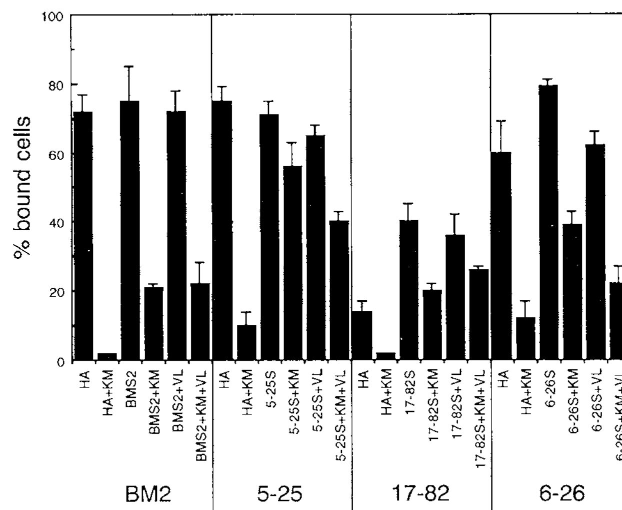

FIG. 5. Effect of antibodies to CD44 and VLA-4 on adhesion of plasma cells to hyaluronic acid (HA) or autologous stromal cells. mAbs used were KM201 (KM) with specificity for CD44 and R1-2 (VL) recognizing VLA-4. Data are presented as mean ± SE for quadruplicate wells. The bone marrow-derived cell line BMS2 and the hybridoma BM2 were again included as positive controls.

binding of plasma cell lines to hyaluronate is routinely less than to feeder cells, suggesting that additional molecules are involved in this adhesion.

In an effort to further characterize the receptors mediating plasma–stromal cell interaction, a panel of mAbs specific for known adhesion molecules was tested for inhibition of plasma cell adhesion. As shown in Fig. 5, the CD44-specific mAb KM201 inhibited binding of plasma cells to autologous feeders. However, in all cases, the inhibition of BM2–BMS2 binding always exceeded that observed between 5-25, 6-26, or 17-82 and their respective feeder cells. A mAb to the integrin VLA-4, either alone or in conjunction with KM201, produced minimal effects and mAbs specific for LFA-1 (CD11a), ICAM-1 (CD54), and Mac-1 (CD11b) failed to inhibit binding of any plasma cell line to its respective feeder.

DISCUSSION

Results of the experiments described above demonstrate that long-term *in vitro* growth of primary plasmacytomas is possible using a feeder layer composed of stromal cells from the initial site of plasmacytomagenesis. The resulting plasma cell lines are indistinguishable from the tumor grown *in vivo* as assessed by isotype of secreted antibody and rearrangement patterns of immunoglobulin loci (Fig. 2). The intimate relationship between primary plasmacytoma and stromal feeders is evidenced by three observations. (*i*) Electron microscopy of the *in vitro* cultures (Fig. 1) suggests that the spatial relationship of the plasma cells and stromal layer is critical since plasma cells are found only on the microvillous surface of the mesothelial cells or the intercellular space between the mesothelial and fibroblastic cells. (*ii*) Early-generation plasma cells do not propagate when separated from feeders by membranes that allow diffusion of soluble materials. (*iii*) Neither supernatants from stromal cell cultures nor IL-6, which has been previously implicated in murine plasmacytoma and human myeloma development (21–23), is sufficient for long-term propagation of these cells. However, the low level of proliferation of later generation plasmacytomas in IL-6 alone (Fig. 3) suggests that some lines may begin to progress to a state of IL-6 dependence and stromal cell independence. The critical role of IL-6 in early plasmacytoma development is demonstrated by the ability of plasma cells to grow on the normally nonsupportive 3T3 line with the addi-

tion of exogenous IL-6 and the inhibition of proliferation on autologous feeders in the presence of anti-IL-6. These observations suggest that at least two elements are critical to the early development of these plasma cell lines, the first being IL-6 and the second being an actual physical interaction with the stromal cells resulting in either a direct or indirect growth stimulus. The adhesion of plasma cells to stromal cell layers appears to be mediated, in part, through interaction of CD44 with its ligand, hyaluronate, as has been shown for the binding of hybridomas to bone marrow stromal cells (11). Adhesion is partially inhibited by mAb to CD44 (Fig. 5) but never reaches 100%, suggesting that the cellular interactions are complex and involve additional cell surface molecules.

Previous studies (9) have clearly demonstrated that primary plasmacytomas cannot be transplanted into normal recipients but grow only in pristane-primed animals. Attempts to grow these tumors in culture have also been unsuccessful. It is thus important to note that all of the primary tumors cultured in these experiments retained their priming dependence for *in vivo* transfer even after as long as 20 months in culture. This result suggests that, even though some lines may undergo neoplastic progression in terms of certain phenotypic characteristics (i.e., IL-6 dependence), stromal cell feeders maintain them in a state requiring pristane priming for successful *in vivo* transplantation. In contrast, *in vivo* passage of primary tumors in primed recipients usually results in a loss of priming dependence after several generations. Such passaged tumors may then be adapted to culture and can be generally divided into two categories—IL-6 dependent *in vitro* or IL-6 independent. IL-6 is present throughout our primary cultures, as it is produced in high quantities by the stromal cells and thus is likely to be one of the critical elements in early plasmacytoma development, yet is insufficient to support growth of early-generation plasmacytomas either alone or as part of a stromal cell supernatant.

Thus, the above data lead to a model allowing a formal definition of the primary neoplastic state in which c-*myc* rearrangement has already occurred but during which "neoplastic" cells are completely dependent on interaction with stromal feeders for further expansion. This dependence may subsequently be lost through any number of successive steps leading first to a partial independence of the stromal layer (although still requiring IL-6) and finally to a stromal or microenvironmental independent stage, which may still require IL-6 and, lastly, autonomous growth. It is noteworthy that a number of similarities can be found between the above murine studies and analyses of human multiple myeloma. Human myeloma lines have proven difficult to establish, but, in a number of instances, such lines have been obtained using conditioned medium (24, 25), skin fibroblasts (26, 27), bone marrow-derived stromal cells (28–31), or IL-6 (28–32). In some cases, these lines appear to "progress" from stromal cell dependence, to soluble factor dependence, to autonomous growth in much the same way as murine plasmacytomas. These similarities suggest that the murine plasmacytoma system provides a useful model for human multiple myeloma and may contribute valuable insights into this disease process.

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