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Establishment of a Human B-Cell Tumor in Athymic Mice[1]

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ABSTRACT

Human B-cell tumors have been established in athymic, BALB/c mice using the EBV-positive Burkitt lymphoma cell line Namalwa. One-hundred-one of 104 animals (97%) developed tumors 10–14 days following s.c. injection of a mixture of 20×10^6 Namalwa and 5×10^6 irradiated human fibrosarcoma (HT-1080) cells. Tumors developed at the site of injection and reached approximately 300 mm² (product of cross-sectional diameters) after 21 days; no metastases were found. Histological analysis showed that tumors consisted solely of lymphoid cells. Immunofluorescence assays demonstrated that while 85% of the tumor cells retained reactivity with the monoclonal B-cell antibody BA-1, 96% retained reactivity with antibody BA-2 and 43% with BA-3. A similar reactivity profile was observed with cultured Namalwa cells. Tumors were passaged serially 10 times without significant change in BA-1, BA-2, or BA-3 reactivity. Indirect immunofluorescence demonstrated that antibody BA-2 reached tumor cells within 2 h following i.p. injection; antigen modulation was not observed. These results demonstrate the suitability of this B-cell model for testing the *in vivo* efficacy and stability of anti-B-cell immunoconjugates.

INTRODUCTION

The transplantation and growth of human tumors in athymic mice has been a very useful tool in the development of reagents and treatment protocols for human cancers. However, the establishment of human leukemias and lymphomas in athymic mice has often been laborious and often unsuccessful, or necessitated establishment of tumors in unusual sites (*e.g.*, eye, brain) (1–2). This situation began to change with the successful establishment of a human acute lymphocytic leukemia cell line in athymic mice (3) and with the use of a human fibrosarcoma cell line to promote tumor acceptance (4). More recently a human T-cell tumor model was developed which will allow evaluation of anti-T-cell antibodies and their immunoconjugates (5).

Use of human T- or B-cell, athymic mouse tumor models should allow determination of the *in vitro* effectiveness of various toxin-, drug-, or radioisotope-containing immunoconjugates synthesized with various coupling methods. Additionally, information may be obtained regarding rates of conjugate clearance and the impact of antiimmunoconjugate antibodies which arise following multiple injections of conjugate (6).

We report here the establishment of a human B-cell tumor model using the EBV-positive Burkitt lymphoma cell line Namalwa (7). This method has produced tumors in 110 of the 115 athymic mice (96%) given s.c. injections of a mixture of Namalwa and irradiated human fibrosarcoma (HT-1080) cells (4). Tumor cells have been passaged serially 10 times without significant change in relevant B-cell surface markers. This tumor model is easily initiated and is reproducible; the potential utility of this model was confirmed by *in vivo* binding of the anti-B-cell monoclonal antibody BA-2 to tumor cells.

MATERIALS AND METHODS

Monoclonal Antibodies. Cells were incubated with the monoclonal anti-B-cell antibodies BA-1 (8), BA-2 (9), BA-3 (10), J5 (11), or B532 (12). Antibody BA-1 is an IgM antibody that reacts with peripheral blood B-lymphocytes, CLL,[3] and most non-T, non-B-ALL, and pre-B-ALL cells (8). BA-2 is an IgG3 antibody recognizing a 24-kilodalton protein (p24) on the surface of most non-T, non-B-ALL cells, on CLL cells, and on some (18%) T-ALL cells (9). Antibody BA-3, an IgG2b κ, reacts with a 100-kilodalton glycoprotein antigen (CALLA) found on the majority of non-T-ALL cells; it also reacts with a small population of normal bone marrow cells. BA-3 reacts with the same or nearly the same eptiope of CALLA as antibody J5 (10). Antibody B532 recognizes an early activation antigen present on human B lymphocytes (12). Antibodies BA-1, BA-2, and BA-3 were obtained as gifts from Hybritech, Inc. (San Diego, CA) and purified from ascites fluids by ammonium sulfate precipitation and protein A-Sepharose chromatography (13). Antibody J5, an IgG2a, was a generous gift from Dr. Jerome Ritz and was also purified from ascites fluid.

Additionally, cells were incubated with the antitransferrin receptor antibody L22 (14), or with the pan-T-cell antibodies T101 (15) or OKT3 (Ortho, Westwood, MA). The murine antibodies MPC-11 (IgG2b) (Sigma Chemical Company, St. Louis, MO) and RPC-5 (IgG2a) were used as irrelevant controls. Antibodies L22, T101, RPC-5, and MPC-11 were all purified from mouse ascites fluid as described above (13).

Tumor Establishment. Four-to-five-week-old female BALB/c *nu/nu* mice, obtained from the Athymic Mouse Facility of the University of California San Diego Cancer Center, were irradiated with 2 Gy weekly for 3 weeks. One week later the animals were injected s.c. in one, or, infrequently, both anterolateral sides, with $2–20 \times 10^6$ Namalwa (7), Nalm-6 (7, 16), or LNPL (17) cells mixed with $5–20 \times 10^6$ irradiated (60 Gy) human fibrosarcoma (HT-1080) (4, 5) cells. Some mice received only Namalwa, Nalm-6, LNPL, or irradiated HT-1080 cells and some mice were used without prior irradiation. Fibrosarcoma cells were irradiated immediately before use. Cells were cultured in RPMI 1640 containing 10% fetal bovine serum and were harvested in mid-log phase growth with viabilities (trypan blue exclusion) exceeding 90%. Initially, mice received injections of 0.5 ml PBS containing the cell mixture. However, the majority of the mice received injections of cells in 0.25 ml of PBS.

Tumor Histology. Twenty-one days after injection of tumor cells, 4 animals with Namalwa tumors were sacrificed and tumor tissue samples obtained. Tumor sites ranged from 192 to 490 mm²; the mean was 317 mm². These samples were fixed in Bouin's solution, imbedded in paraffin, sectioned, and stained with eosin and hematoxylin. A representative tissue section is shown in Fig. 1.

Immunofluorescence Studies. Freshly excised tumor was rendered into single-cell suspensions and the cells incubated with various individual monoclonal antibodies to determine the cell-surface phenotype of the tumor cells. Twenty-five μl of cells at 50×10^6/ml was incubated for 30 min at 4°C with 50 μl of test antibody at a final antibody concentration of 10 μg/ml. The cells were then washed twice and incubated with affinity-purified, fluorescein-conjugated, goat anti-mouse antibody (TAGO, Burlingame, CA) for 30 min at 4°C and the cells again washed twice. The cells were then fixed in 1% formaldehyde, filtered, and analyzed using an Ortho Cytofluorograf (Ortho, West-

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[3] The abbreviations used are: CLL, chronic lymphocytic leukemias; PBS, 0.01 M potassium phosphate buffer saline, pH 7.4, containing 0.15 M sodium chloride; FITC, fluorescein isothiocyanate; MIF, mean intensity of fluorescence; CALLA, common acute lymphoblastic leukemia antigen.

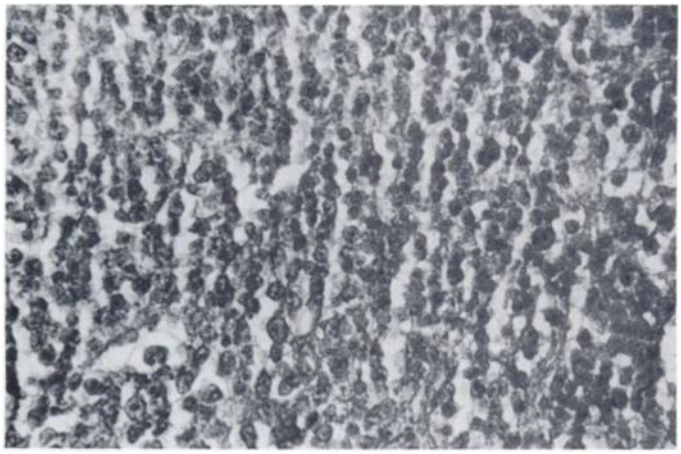

Fig. 1. H and E-stained tissue section of Namalwa tumor from an athymic mouse; × 125.

Table 1 *Tumor formation using LNPL, Nalm-6, or Namalwa cells*

Animals were irradiated and injected as described in "Materials and Methods." Following injection the mice were observed semiweekly for tumor formation. Irradiated HT-1080 cells did not grow in culture and alone did not produce tumors in irradiated athymic mice.

No. animals injected	Cells × 10^6	HT-1080 cells × 10^6	No. tumors produced
LNPL cells			
10	2[a]	0	0
10	2[a]	5	0
Nalm-6 cells			
12	20[b]	0	0
4	2[b]	5	0
12	5[b]	5	0
26	20[b]	5	0
10	20[b]	20	0
2	32[b]	8	0
Namalwa cells			
20	20[c]	0	15 (75)[d]
3	2[c]	5	0
9	5[c]	5	6 (67)
6	15[c]	5	5 (83)
104	20[c]	5	101 (97)
5	20[c]	20	4 (80)

[a] LNPL cells.
[b] Nalm-6 cells.
[c] Namalwa cells.
[d] Numbers in parentheses, percentage of tumors produced within each group of animals.

wood, MA) and 2150H computer. Tumor cells were also cultured *in vitro* for 5 days and incubated with the panel of antibodies as described above.

***In Vivo* Binding of BA-2.** Twenty-three tumor-bearing, athymic mice were injected i.p. with 200 μg of BA-2 and sacrificed at time zero or after 2, 4, or 24 h. Tumors were removed, rendered into single-cell suspensions, and assayed for BA-2 binding by incubating the cells directly with affinity-purified, fluorescein-conjugated, goat anti-mouse antibody or, to determine *in vivo* modulation and saturation values, with the irrelevant control antibody MPC-11 followed by incubation with the FITC-conjugated secondary antibody. Additionally, aliquots of cells from each time point were also incubated *in vitro* with additional BA-2, washed, and then incubated with the secondary antibody to test for the presence of residual p24 antigen.

RESULTS

A summary of the conditions used to establish Namalwa tumor xenografts in athymic mice is presented in Table 1. Mice which had not been irradiated prior to injection did not develop tumors, regardless of whether Namalwa, Nalm-6, or LNPL cells were used alone or in combination with irradiated HT-1080 cells (data not shown). Similarly, irradiated mice injected solely with irradiated fibrosarcoma (HT-1080) cells (not shown) or the EBV-negative cell lines Nalm-6 or LNPL did not develop tumors (Table 1). Lastly, irradiated mice injected with mixtures of irradiated HT-1080 cells plus Nalm-6 or LNPL cells also did not develop tumors (Table 1). Because initial experiments with Nalm-6 and LNPL cells were negative, they were not studied further.

Table 2 *Cell surface phenotype of cultured Namalwa and HT-1080 cells*

Cell lines were cultured in RPMI 1640 containing 10% fetal bovine serum and harvested in mid-log phase growth; viabilities exceeded 90% as determined by trypan blue exclusion. The cells were incubated with various antibodies as described in the "Materials and Methods" section; MPC-11 is an irrelevant IgG2b control antibody. Results similar to those shown for MPC-11 were obtained when the irrelevant antibody RPC-5 (IgG2a) was used (not shown). The percentage of positive cells represents the percentage of cells binding the test antibody. The total MIF is the MIF for all cells while the positive MIF is the value assigned to only the positive cells. While the data shown are from one experiment similar results were obtained on at least three other occasions.

	Antibody	% Positive cells	Total MIF	Positive MIF
HT-1080	MPC-11	1	9	38
	J5	98	90	92
	BA-1	1	9	33
	BA-2	19	17	37
	BA-3	95	73	76
	B532	1	10	35
	OKT-3	1	10	38
	T101	2	9	42
	L22	17	18	40
NAMALWA	MPC-11	1	7	21
	J5	87	30	33
	BA-1	87	55	62
	BA-2	86	43	36
	BA-3	32	14	21
	B532	8	9	31
	OKT-3	2	7	19
	T101	2	7	19
	L22	17	12	32

In contrast to the results obtained with Nalm-6 and LNPL cells, irradiated mice injected with Namalwa cells alone or in combination with irradiated HT-1080 cells developed tumors readily. While 15 out of 20 irradiated mice (75%) developed s.c. tumors at the site of injection when injected solely with 20 × 10^6 cultured Namalwa cells, 97% (101/104) of the mice given 20 × 10^6 Namalwa cells plus 5 × 10^6 irradiated HT-1080 cells produced s.c. tumors, also at the site of injection, within 10–14 days. No difference in tumor formation was noted between mice injected with cell mixtures suspended in 0.25 or 0.50 ml of PBS. Namalwa tumors grew rapidly, reaching 200–400 mm^2 (product of cross-sectional diameters) within 3 weeks. No metastases were noted during gross anatomical examination of lymph nodes, livers, or spleens from 6 tumor-bearing mice.

When dissected cross-sectionally, tumors were firm and beige in color. Necrosis was not observed unless the tumors were larger than approximately 400 mm^2 in size. When observed, necrosis was confined to the exterior surfaces of the tumor. Tumors consisted entirely of lymphoid cells when stained with eosin and hematoxylin (Fig. 1); HT-1080 cells, which presumably would have been large with eosinophilic cytoplasm (4, 5) were not seen. Analysis of sections from tumors and surrounding tissues by immunoperoxidase staining techniques was not attempted because similar studies, conducted previously on tissue samples from mice bearing MOLT-4 human T-cell tumors (5), were unsuccessful due to endogenous mouse immunoglobulin.[4]

Table 2 shows the cell-surface reactivity of cultured HT-1080 and Namalwa cells with a panel of 9 different antibodies. Similar reactivity patterns were obtained when both cell lines

[4] R. O. Dillman, D. E. Johnson, D. L. Shawler, S. E. Halpern, J. E. Leonard, and P. L. Hagan, unpublished results.

were incubated with antibodies J5 and BA-3, and with the antitransferrin receptor antibody L22. Surprisingly, antibody BA-3 reproducibly reacted more strongly with HT-1080 cells than with Namalwa cells. Antibodies BA-1 and BA-2 reacted more strongly with Namalwa cells than with HT-1080 cells. The B-cell antibody B532 showed no reactivity with HT-1080 cells and only minimal reactivity with cultured Namalwa cells. Finally, the anti-T-cell antibodies T101 and OKT-3 did not exhibit reactivity with either cell line.

The results shown in Table 3 demonstrate that antibody BA-2 reached the Namalwa tumor cells within 2 h following a single i.p. injection of 200 μg of antibody in tumor-bearing mice. The values shown were obtained following incubation of tumor cells with the irrelevant antibody MPC-11, followed with the secondary antibody, and were used in calculation of *in vivo* modulation and saturation values (18). They did not differ significantly from those obtained after incubating the tumor cells directly with the secondary antibody (data not shown). Tumor cells taken from mice sacrificed immediately following injection of BA-2 (time zero) showed no antibody binding, as demonstrated by the absence of fluorescence following incubation with FITC-conjugated goat anti-mouse antibody (not shown) or after incubation with control antibody MPC-11 followed by the FITC-conjugated secondary antibody (Table 3). Both the percentage of positive cells and the total MIF of these cells increased following incubation *in vitro* with additional BA-2.

Cells taken from tumor-bearing mice 2 h after injection of BA-2 exhibited surface-bound BA-2 antibody as indicated by the high percentage of positive cells and the increased total MIF value. Again, similar results were obtained when tumor cells were incubated directly with the FITC-conjugated secondary antibody (not shown). Moreover, 2 h after injection of BA-2 virtually all of the antigen binding sites were saturated, as indicated by the absence of increase in the percentage of positive cells following incubation *in vitro* with additional BA-2 antibody (Table 3). The *in vivo* saturation values shown are quotients obtained by dividing the percentage of positive cells determined following incubation with the irrelevant antibody MPC-11 by the percentage of positive cells obtained following incubation *in vitro* with additional BA-2 (18). Similar results were obtained with Namalwa cells analyzed 4 and 24 h after injection. When the average total MIF values or the means of the percentage of positive cells, each obtained from three separate experiments, were plotted *versus* time they both produced hyperbolic profiles (not shown). Maximum values for each measure of antibody binding were attained at 2 and 4 h, respectively. Antigen modulation was not detected, even after 24 h (Table 3).

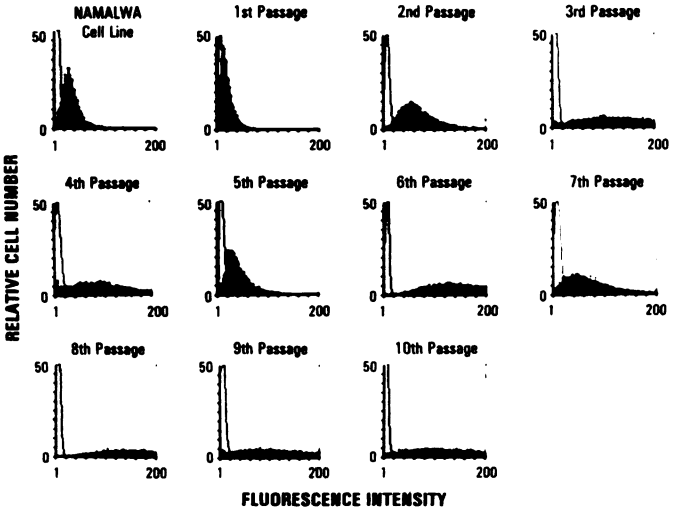

Fig. 2. Histograms showing the immunofluorescence profiles for cultured Namalwa cells and passaged Namalwa tumor cells incubated with the anti-p24 antibody BA-2. *Ordinate*, relative cell number; *abscissa*, intensity of fluorescence. First passage cells were derived from tumors established from cultured Namalwa cells and passaged subsequently into a second mouse.

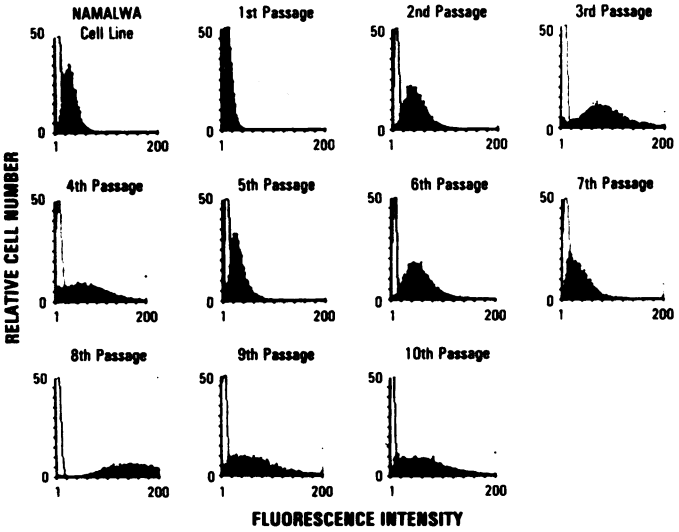

Fig. 3. Histograms showing the immunofluorescence profiles for cultured Namalwa cells and serially passaged Namalwa tumor cells (passages 1 through 10) incubated with the anti-CALLA antibody J5 and subsequently with a fluorescein-conjugated antimouse secondary antibody. *Ordinate*, relative cell number; *abscissa*, fluorescence intensity.

Table 3 *In vivo uptake of antibody BA-2 by Namalwa tumor cells*

Five-to-seven tumor-bearing mice for each time point were injected i.p. with 200 μg of BA-2 and sacrificed at the times shown. Tumor sizes ranged from 169 to 306 mm². Cells incubated with only the FITC-conjugated secondary antibody produced results similar to those shown following incubation with the irrelevant antibody MPC-11 and subsequently with the secondary antibody. The modulation and *in vivo* saturation values were calculated as previously described (18). The results shown are the means of three separate experiments; standard errors for percentage of positive cells and for total MIF values ranged from 1 to 12.

Cells	MoAb	% Positive cells	Total MIF	*In vivo* Modulation	*In vivo* Saturation
Namalwa cells	MPC-11	3	7	na	na
	BA-2	94	115	na	na
Tumor cells					
0 h	MPC-11	18	15	na	0.19
	BA-2	94	108		
2 h	MPC-11	95	45	0.94	1.02
	BA-2	93	102		
4 h	MPC-11	86	59	0.82	0.96
	BA-2	90	91		
24 h	MPC-11	88	48	0.89	0.92
	BA-2	96	98		

Established Namalwa tumor cells were easily passaged in the absence of HT-1080 cells. Over a period of roughly 12 months 58 of 60 irradiated animals (97%) injected with 15×10^6 passaged Namalwa tumor cells developed tumors. Tumor sizes ranged from 288 to 530 mm². When tumor cells were analyzed for the presence of the p24 and CALLA antigens at least 85% of the cells incubated with antibodies BA-2 or J5 were positive for antibody binding. While in some instances the mean intensity of fluorescence values were low (*e.g.*, 35), in most instances the MIF values exceeded 100. Expression of p24 antigen was retained in passaged tumor cells (Fig. 2) and the MIF values exceeded those determined for cultured Namalwa cells. CALLA expression was also retained in serially passaged tumor cells (Fig. 3) as indicated by binding of antibody J5. Similar results were obtained with passaged tumor cells incubated with antibodies BA-1, BA-3, and L22 (not shown).

DISCUSSION

Using previously established techniques (5) we have developed a human B-cell tumor model in athymic mice employing the EBV-positive Burkitt's lymphoma cell line Namalwa (7). Subcutaneous tumors were easily and reproducibly established in irradiated animals. Using 15–20 × 10^6 cultured Namalwa cells and 5 × 10^6 irradiated HT-1080 cells we were able to successfully establish primary tumors in 96% (110/115) of the animals tested. The best results were obtained using 20 × 10^6 Namalwa cells plus 5 × 10^6 irradiated HT-1080 cells. Tumors generally were palpable within 10 days of injection. When fibrosarcoma cells were omitted primary tumors were established in 75% (15/20) of the irradiated animals. No tumors were produced if the mice were not irradiated prior to injection, regardless of the combination HT-1080 and Namalwa cells used. Thus irradiation appears to be required for efficient tumor establishment, and may eliminate residual natural killer cell activity (4).

While the presence of irradiated HT-1080 cells was not an absolute requirement for Namalwa tumor formation, the incidence of tumor formation was increased markedly by the presence of irradiated HT-1080 cells (96 *versus* 75%). This suggests that the irradiated fibrosarcoma cells secrete an angiogenesis or conditioning factor (or factors) required for tumor establishment (19, 20). This conclusion is supported by a recent report by Picard, *et al.* (21) in which they demonstrated that coinjection of treated fibroblasts with a number of cell lines facilitated tumor take, as did injection of cell lines suspended in fibroblast-conditioned medium. Once the Namalwa cells had been established in a conditioned environment they were apparently able to produce sufficient quantities of a B-cell growth factor, or factors, by an autocrine mechanism (22), and could be passaged from mouse to mouse in the absence of irradiated fibrosarcoma cells. Most importantly, these passaged tumor cells continued to express the p24 and CALLA antigens as well as those recognized by antibodies BA-1 and BA-3.

As shown in Fig. 2, reactivity of antibody BA-2 with passaged tumor cells remained at least as high if not higher than reactivity with cultured Namalwa cells, with the possible exception of passage 1, throughout all 10 passages of the tumor cells. Similar results were obtained with antibody J5 (Fig. 3). Fluctuations in fluorescence intensity of antibody binding to passaged cells may be due in part to relative tumor size, and thus the number of viable cells, and variations in gate settings on the cytofluorograf.

The ease of tumor initiation and the continued expression of the p24 and CALLA antigens make this a suitable model for evaluating the efficacy and stability of anti-B-cell immunoconjugates *in vivo*. Frank reductions in the size of established Namalwa tumors using anti-B-cell immunoconjugates may require multiple injections of A-chain-linked conjugates or perhaps the use of homologous ricin A-/B-chain immunotoxins (23, 24). The use of multiple injections of immunoconjugate raises the prospect of host production of antitoxin antibody. Such antibodies have been detected 26 days after initiation of a series of injections of anti-Thy 1.1-ricin A-chain conjugate (6). This model provides a setting for assessing the full impact of these antibodies on the antitumor effects of various immunoconjugates. Finally, the *in vivo* stability of immunoconjugates synthesized with reducible or nonreducible cross-linking reagents could be evaluated using this model system. While some work has been published in this regard (6, 25, 26), the results are contradictory and bear further study. The Namalwa B-cell model described above is easily established and reproducible, and provides a means for studying these important questions.

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