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Generation of human monoclonal antibodies by transformation of lymphoblastoid B cells with ras oncogene*

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Abstract

Human monoclonal antibodies (hu-mAbs) of predetermined specificity and isotype are potentially important for a variety of applications, including therapy and diagnosis. Their efficient generation, however, is still hampered by technical difficulties. Even the most established approaches to the generation of hu-mAbs, i.e., B cell immortalization by Epstein-Barr virus (EBV) and/or fusion with appropriate myeloma cell lines, are characterized by a relatively low efficiency. It has been shown that expression of activated Ha- or N-ras oncogenes causes the malignant transformation and plasmacytoid differentiation of EBV-immortalized lymphoblastoid cell (LC) lines, suggesting that activated ras oncogenes can convert LC lines into effective hu-mAb producers. We have used retroviral vector-mediated gene transfer to introduce an activated Ha-ras (v-ras) oncogene into four distinct LC lines producing hu-mAbs of different classes (IgM and IgG) and specificities (to human insulin, human thyroglobulin and rabies virus glycoprotein). The cloning efficiency and antibody secretion of these ras-transformed LC (ras-LC) lines were compared with those of the hybrid LC (hyb-LC) lines generated by fusing the same parental LC lines with the Ig non-secreting F3B6 human-mouse hybrid cells. ras-LC lines were comparable to their hybrid counterparts in either parameter tested. This, together with the relatively higher efficiency of the method, suggests that ras transformation may constitute a valid alternative to the currently available technologies for hu-mAbs production from LC lines.

Keywords

Monoclonal antibody; human; Lymphoblastoid cell line; Ras oncogene; Retroviral vector

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Introduction

hu-mAbs represent potentially invaluable tools for diagnostic, prophylactic and therapeutic procedures (Larrick and Bourla, 1986) and display important advantages over their murine counterparts (Dilman, 1990; Goldenberg, 1990; Vaickus and Foon, 1991): (i) they are immunologically tolerated, while mouse IgG elicit an immune response which prevents prolonged therapeutic use; (ii) they are endowed with antigen specificities more representative of those physiologically occurring *in vivo*; and (iii) they display effective *in vivo* biological functions, including enhanced antibody-dependent cellular cytotoxicity (ADCC), due to a higher affinity for their receptors on effector cells of the immune system (Carson and Freimark, 1986).

Currently, three main approaches are used to generate hu-mAbs: (i) isolation of genetically engineered antibodies from recombinant expression libraries (Sasthy et al., 1989; Huse et al., 1989), (ii) construction of chimeric 'humanized' mouse mAbs (Morrison, 1992), and (iii) direct immortalization of human B lymphocytes (James and Bell, 1987). All these strategies have been successfully used. Nevertheless, each has some limitations, such as the potential lower affinity of library-derived mAbs (Caton and Koprowski, 1990; Winter and Milstein, 1991), the residual immunogenicity of humanized antibodies (Bruggeman et al., 1989), and the relative instability and low rate of mAb production of LC *in vitro* established by various methods (Roder et al., 1986; James and Bell, 1987).

It has been reported that the expression of activated ras oncogenes such as v-ras or a mutated N-ras allele, lead to malignant transformation of LC lines, resulting in increased clonogenicity in soft agar and tumorigenicity in nude mice, as well as differentiation into plasmacytoid cells capable of enhanced antibody secretion (Seremetis et al., 1989). Based on these findings, we have used ras oncogene transformation to overcome two major limitations of LC lines as hu-mAb producers, namely, poor clonogenicity and low rate of antibody secretion. Constitutive expression of a v-ras oncogene by LC lines, previously selected for antigen specificity, was achieved by retroviral-mediated gene transfer. In each case, the potential for mAb production of ras-LC lines was similar to that of the corresponding hyb-LC line generated by fusing the same parental LC with an immortal partner, suggesting that ras-LC lines acquire all the features necessary for efficient and sustained mAb production.

Materials and methods

Cell lines

Human B lymphocytes were purified from the peripheral blood of healthy subjects and transformed with EBV (Larrick et al., 1988; Ikematsu et al., 1992a). LC lines were selected for the production of IgG antibodies to rabies virus glycoprotein (Ueki et al., 1990), IgM and IgG antibodies to human insulin (Casali et al., 1990) and IgM antibodies to human thyroglobulin (Sanz et al., 1989). Monoclonal LC lines were generated by at least three sequential subculturing steps. The produced mAbs have been previously characterized for their binding activities and dissociation constants (K_d) for various antigens (Sanz et al., 1989; Ueki et al., 1990; Casali et al., 1990; Ikematsu et al., 1992a).

Generation of continuous monoclonal hybrid cell lines

Each monoclonal antigen-specific LC line was stabilized by fusion with F3B6, an Ig non-secretor, HAT-sensitive and ouabain-resistant human-mouse hybrid cell line, as described (Nakamura et al., 1988; Larrick et al., 1988; Casali and Notkins, 1989; Ikematsu et al., 1992a).

Generation of continuous monoclonal transformed cell lines by gene transfer

For transduction of the v-ras oncogene into LC lines, we used the retroviral vector produced by the cell line ψ 2-AM Ras Zip6 cl.5 (Dotto et al., 1985). These cells produce a retrovirus whose genome carries the viral Harvey-ras oncogene (v-Ha-ras) and the neo-resistance gene. As a control, we used a vector containing only the neo-resistance gene produced by the cell line PA317 N2 (Armentano et al., 1987). Antigen-selected LC lines were infected with retrovirus by incubation in undiluted viral stock in the presence of 8 μ g/ml polybrene (multiplicity of infection of 3:1), for two 3 h rounds. The two rounds of infection were separated by an interval of 16 h. In each infection round, 10×10^6 cells were resuspended in the virus-containing medium, previously passed through a 0.45 μ m filter, and incubated at 37°C in 5% CO₂ with frequent and gentle agitation. Cells were eventually plated at 1×10^6 cells/ml in fresh Iscove's modified Dulbecco's medium (Gibco Laboratories, Grand Island, NY). 24 h later, they were selected for antibiotic resistance in medium containing 0.4 mg/ml (active dose) of geneticin (Gibco Laboratories, Grand Island, NY).

Northern blot hybridization analysis

Total RNA was extracted by the guanidine isothiocyanate method (Chirwin et al., 1979). For Northern blot analysis, RNA samples were fractionated by electrophoresis in 0.9% agarose/2.2 M formaldehyde and then transferred to nitrocellulose filters. Hybridization analysis was performed in 50% formamide, 3 $\times$ SSC, 10% dextran sulfate, 5 $\times$ Denhardt's solution, 0.5% SDS and 100 μ g/ml of salmon sperm DNA at 37°C for 16 h. Filters were rinsed four times in 0.2 $\times$ SSC/0.5% SDS at 37°C and then washed in 0.2 $\times$ SSC/0.5% SDS at 60°C for 2 h. DNA probes were ³²P-labeled by the random primer oligolabelling method (Feinberg and Vogelstein, 1983).

DNA probes

The ras probe was represented by a 0.8 kb BamHI fragment containing v-Ha-ras sequences derived from the ras-zip 6 plasmid (Dotto et al., 1985). The GAPDH probe, was a 1.4 kb PstI fragment corresponding to the rat glyceraldehyde-3-phosphate dehydrogenase cDNA subcloned into pGem 3 (Grignani et al., 1990).

Culture of ras-, neo- and hyb-LCs and determination of their cloning efficiency

ras-, neo- and hyb-LCs were distributed at 400, 300, 150, 75, 37, 18, 9, 4, 2, 1 cells per microculture in 96 round-bottomed well plates in absence of feeder layer. After 2 weeks, the microcultures were examined for the presence of growing cells, as reported (Nakamura et al., 1988; Inghirami et al., 1988; Ikematsu et al., 1992a) and for the amounts of mAb produced, as described (Ikematsu et al., 1992a). The frequency of outgrowing cells (clonogenicity) was calculated by statistical analysis according to Poisson distribution by

plotting the fraction of negative microcultures (seeded microwells containing no growing cells) versus the cell dose (number of seeded cells/well), as previously described (Larrick et al., 1988; Nakamura et al., 1988; Inghirami et al., 1988; Ueki et al., 1990; Ikematsu et al., 1992a).

Titration of total antibody and specificity

IgM and IgG concentrations were measured using appropriate ELISAs, as previously described (Nakamura et al., 1988; Casali et al., 1990; Ueki et al., 1990; Ikematsu et al., 1992a). Reference binding curves were constructed using purified IgM and IgG. For titration of IgM and IgG to various antigens, plates were coated with purified human recombinant insulin, purified human thyroglobulin or purified rabies virus glycoprotein (Sanz et al., 1989; Nakamura et al., 1988). Specific horseradish peroxidase- or alkalyne phosphatase-conjugated affinity-purified goat antibodies were used to detect IgH and L chains. Alkaline phosphatase-conjugated specific mouse mAbs were used to detect the different IgG subclasses. Substrate conversion was measured as absorbance at 492 or 415 nm using an automated ELISA reader. The lower limit of sensitivity of the ELISA was 5 ng Ig/ml.

Results

Generation of ras-LC lines

For this study, four previously characterized LC lines, representative of different Ig classes and antigen-specificities were selected. The continuous LC lines were generated by sequential subculture and have been previously characterized for the binding activity and affinity for antigen of the corresponding mAbs (Nakamura et al., 1988; Sanz et al., 1989; Casali et al., 1990; Ueki et al., 1990; Ikematsu et al., 1992b,c). Two of these cell lines (cl 65 and cl 91) produced IgM and IgG mAbs to human insulin; the third cell line (cl 25) produced an IgM mAb to thyroglobulin; the fourth cell line (cl 107) produced IgG1 mAb to rabies glycoprotein (Table I).

Each LC line was infected by the amphotropic retroviral vector carrying the v-ras oncogene and the neo gene as a selection marker. The vector containing the neo-resistance gene alone was used to generate control-infected LC (neo-LC) lines. After infection and antibiotic selection, ras- and neo-LC lines were studied for v-ras expression by Northern blot analysis. Representative data relative to two clones, LC cl 91 and LC cl 107, are shown in Fig. 1. The 5.4 kb message, present exclusively in ras-LC lines and corresponding to v-ras gene transcription driven by the MuLV-LTR promoter, documents the infection and v-ras expression in the ras-LC lines.

Comparative analysis of clonogenicity of ras-DC and hyb-LC lines

ras-LC, neo-LC and hyb-LC obtained from each original LC line were distributed in limiting dilution conditions, in absence of feeder layer, and, after 2 weeks, were analyzed for the frequency of outgrowing cells. As shown in Table I, the outgrowing rate of ras-LC lines was 7 (cl 107, ras-LC(a))- to 55 (cl 91, ras-LC(a))-fold greater than that of neo-LC lines. In general, it was within the same order of magnitude as that of the corresponding hyb-LC line. In two cases, LC cl 25 and cl 65, the frequencies of outgrowing cells were closely

comparable in ras- versus hyb-LC. In one case, cl 107, the two independently generated ras-LC lines were both about four-fold more clonogenic than their respective hyb-LCs. In the last case, cl 91, one ras-LC line (clone a) was comparable to the corresponding hyb-LC line, whereas the second (b) was about 30 fold less clonogenic. Thus, ras transformation significantly enhances the clonogenicity of LCs to levels at least comparable to those characteristic of cloned hyb-LCs.

m-Abs secreted by ras-LC and hyb-LC lines

The titers of mAbs secreted by neo-, ras- and hyb-LC lines were determined by appropriate ELISAs. As shown in Table I, the mean amounts of mAbs secreted by ras-LC lines were 4–36-fold greater than those secreted by neo-LC lines. In two cases, cl 25 and cl 65, ras-LC lines display titers of mAb secreted that approached those obtained from hyb-LC lines; in one case, cl 107, two independently generated ras-LC lines produced both markedly greater amounts of mAbs than their respective hyb-LC lines; in the last case, cl 91, consistent with their respective clonogenicity rates, one ras-LC (a) secreted an amount of mAb comparable to that of the selected hyb-LC, while the other (b) did not. In each case, increased production of mAbs by ras-LC lines was associated with preservation of antigen-specificities (data not shown).

Discussion

Constitutive expression of v-ras oncogenes leads to malignant transformation of LC lines, as suggested by the high clonogenicity of these cells in soft agar and their acquired tumorigenicity in nude mice (Seremetis et al., 1989). In addition, constitutive expression of ras oncogene by B cells is associated with acquisition of a plasmacytoid phenotype displaying specific cell surface antigens and enhanced antibody secretion (Seremetis et al., 1989). These findings prompted us to use ras transformation as a powerful tool for the generation of consistently highly proliferating and secreting hu-mAb producing cell lines.

The selection of a B cell committed to the production of an antibody of predetermined specificity and isotype depends, first of all, on prior efficient culture of a polyclonal B lymphocyte population. This in general is achieved by transforming freshly isolated B lymphocytes with EBV or hybridizing them with a fusion partner, e.g., a myeloma cell line. EBV infection constitutes a more efficient method for initial B cell expansion and selection, yielding an up to 5×10^{-2} incidence of outgrowing transformed cells, in contrast to direct B cell-myeloma fusion, resulting in general in 10^{-5} – 10^{-6} fusion events. EBV infection, however, falls short of endowing B lymphocytes with continuous and effective, i.e., high rate, growth potential. Continuous and high rate growth are critical for successful subculturing and formal cloning of selected B lymphoblasts. In fact, continuous and steady growth, suitable for effective subcloning, of LC lines have been best achieved by their fusion with a preconstructed Ig non-secretor human-mouse fusion partner. Using this procedure, several high affinity hu-mAbs with different antigen specificities have been generated (Nakamura et al., 1988; Casali and Notkins, 1989; Ueki et al., 1990; Casali et al., 1990). Although this method is reliable and reproducible, it suffers from the relatively low rate of fusion events (Roder et al., 1986; James and Bell, 1987; Carson and Freimark, 1989).

The novel approach proposed here uses LC lines, but substitutes somatic cell hybridization with ras transformation. The efficacy of this strategy results from the highly efficient gene transfer mediated by retroviral vectors (Miller and Rosman, 1989). Retroviral vector infection is easy to perform and leads to the generation of ras-LCs within 2–3 weeks. Most notably, this procedure is characterized by an efficiency several orders of magnitude greater than cell hybridization (approximately 10^{-1} versus 10^{-5}), (Hwang and Gilboa, 1984; James and Bell, 1987) and, as shown in this study, yields plasmacytoid elements that display degrees of clonogenicity and mAb secretion at least comparable to those of somatic cell hybrids. It should be emphasized that the present results have been obtained using relatively unselected ras-LC lines, suggesting that repeated subclonings, as routinely performed to isolate optimal hyb-LC clones, are likely to generate ras-LC clones with even greater clonogenic and secretory potentials. Thus, the approach outlined here constitutes a valid alternative over the currently available methods for human B cell immortalization, cloning and mAb production. Additional testing of the ras transformation method, proposed here, should be performed to explore the full potential of its applicability to the generation of hu-mAbs. Simultaneous infection of B lymphocytes with EBV and a ras-carrying retroviral vector followed by in vitro selection for antigen specificity should avoid the time consuming steps involved in cloning and characterization of poorly clonogenic LC lines and further simplify the overall procedure of hu-mAb generation.

Abbreviations

hu-mAb	human monoclonal antibody
EBV	Epstein-Barr virus
LC	lymphoblastoid cell
neo	neomycin
hyb	hybridoma
ras-LC	ras-transformed LC
neo-LC	control-infected LC
hyb-LC	LC fused with a somatic cell hybridization partner

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Fig. 1.

Top panel: Northern blot analysis of ras mRNA expression by LC lines. Total RNA (16 μ g) from parental (lanes 1), neo-(lanes 2) and ras-LC lines (lanes 3) of two representative clones, LC cl 107 and LC cl 91, was hybridized with a BamHI fragment derived from plasmid ras-zip 6. Hybridization of the same filter to a rat glyceraldehyde-3-phosphate dehydrogenase probe is shown as a control for the amounts of RNA analyzed. *Bottom panel:* Schematic representation of the ras-zip 6 retroviral vector and the probe.

CLONING EFFICIENCY OF AND ANTIBODY SECRETION BY neo-, ras- AND hyb-LC LINES

TABLE I

		LC cl 25 (IgM, κ , to thyroglobulin)			LC cl 91 (IgG1, κ , to insulin)		
		neo-LCs	ras-LCs	hyb-LCs	neo-LCs	ras-LCs (a) ^d	hyb-LCs
Frequency of outgrowing cells ^b		1:610	1:25	1:30	<1:1000	1:18	1:400
mAb titer ^c	max	90	275	800	17.5	875	250
	mean	35	200	500	7.5	500	50
	min	17.5	125	350	1.75	250	17.5
							250

		LC cl 65 (IgM, κ , to insulin)			LC cl 107 (IgG1, λ , to virus rabies gp)		
		neo-LCs	ras-LCs	hyb-LCs	neo-LCs	ras-LCs (a) ^d	hyb-LCs
Frequency of outgrowing cells ^b		1:700	1:37	1:40	1:2,500	1:350	1:312
mAb titer ^c	max	850	3,300	5,200	40	1,000	1,200
	mean	290	1,200	5,000	38	460	920
	min	6	2,560	4,800	35	230	460
							39

^a Different samples of the same LC line were independently infected with the v-ras oncogene.^b The frequency of outgrowing cells was evaluated by statistical analysis according to Poisson distribution (see materials and methods section).^c Antibody titers are expressed in ng/ml and represent the mean value of 24 microcultures tested after a 2 week culture at the time of the assessment of the frequency of outgrowing cells. Reported values are relative to 150 cells.