Production of Hybridomas Secreting Monoclonal Antibodies against the Lymphokine Osteoclast Activating Factor

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ABSTRACT The human lymphokine osteoclast activating factor (OAF) is thought to be involved in several bone-destroying diseases. The current studies were designed to produce monoclonal antibodies against OAF for use in subsequent design of immunoassays for OAF in clinical samples. Spleen cells from mice immunized with purified human OAF were hybridized with mouse plasmacytoma cells in vitro to yield hybridomas. Several clones of these hybridomas secreted into the culture medium antibodies, which neutralized the biological activity of OAF at dilutions as high as 1:100,000 relative to the initial culture medium. These antibodies did not interfere with the activities of parathyroid hormone in the same systems. These results represent the first report of monoclonal antibodies against a human lymphokine, and validate the concept that hybridoma production is a useful technique for developing antibodies against weak or scarce antigens.

INTRODUCTION

The lymphokine osteoclast activating factor (OAF)[1] is known to stimulate bone resorption in vitro (1). It seems likely that OAF is involved in one or more diseases that are characterized by chronic accumulation of inflammatory cells in conjuction with localized bone destruction, such as myelomas (2, 14), periodontal disease (3), or other lymphoproliferative diseases (4). Definite demonstration that OAF is involved in the bone destruction of any of these diseases would furnish useful information in terms of their treatment and etiology. Such definite demonstration has not yet been achieved. Specific antibodies against OAF would appear the most straightforward means for developing immunoassays and immunocytochemical tests that would allow direct testing for OAF in clinical samples. Unfortunately, OAF is a small peptide of ≅9,000 daltons (5) that has proven not sufficiently immunogenic to produce useful titers of antisera in animals immunized by classical techniques. Recently, however, procedures have been developed by which clones of plasma cells, activated to produce specific antibodies by immunization in vivo, can be isolated and "immortalized" by somatic cell hybridization with long-term plasmacytoma cell lines (6–8). An important corollary of these techniques for the present studies is that, if sufficiently sensitive screening assays are available, hybrid plasma cell clones (hybridomas) can be isolated whose antibodies are specific for substances that are weak antigens or minor components of the immunizing mixture. Previous examples of such specificity have been the isolation of monoclonal antibodies against histocompatibility antigens (6) or tumor antigens (7), which were minor components of very complex mixtures (e.g., whole cells). The specific hybridoma clones, once isolated, can be propagated as mass cultures or as tumors in mice (8) to furnish significant quantities of monoclonal, monospecific antibody against the antigen of choice. In this report, we describe the isolation of hybridomas that secrete monoclonal antibodies against OAF.

METHODS

Assessment of anti-OAF activity. Culture media from cells hybridized as described below were pooled over several changes of medium, and then concentrated by ultrafiltration. Immunoglobulins were isolated by passing the concentrated

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[1] *Abbreviations used in this paper*: cAMP, cyclic AMP; HAT, culture medium consisting of MEM with 0.1 mM hypoxanthine, 0.01 mM aminopterin, and 0.03 mM thymidine; MEM, Eagle's minimal essential medium; OAF, osteoclast activating factor; PTH, parathyroid hormone.

medium over columns of DEAE-cellulose equilibrated with 20 mM Tris-HCl, pH 7.8. The wash-through fractions were collected and used for assay of antibody activity. Medium that had not been used for culture, as well as medium containing immunoglobulins secreted by the parental plasmacytoma cell line, was also treated in the same way to serve as control. To assay for antibody activity, the isolated immunoglobulin fraction was diluted to the indicated extent (relative to initial culture medium volume) in Eagle's minimal essential medium (MEM) containing either 3 ng/ml purified OAF or 30 ng/ml bovine parathyroid hormone as indicated. The samples were incubated at 4°C overnight to allow antigen-antibody interaction, and then were added to cultured mouse cranial bones. The concentration of cyclic (c) AMP was determined in the bones after a 5-min incubation as described (9). Alternatively, the bone-resorbing activity of the incubated samples was tested by adding aliquots at the indicated dilutions to the culture medium of mouse cranial bones, which were then maintained in organ culture for a further 72 h. At the end of this culture period, the release of calcium from the cultured bones was measured (10). Whichever assay was used, anti-OAF activity was estimated as the decrease in OAF activity caused by incubation with hybridoma supernates. Each incubated sample was tested at three or more dilutions to construct a dose-response curve for the biological activity in the sample. The percentage of recovery of activity was estimated by means of a computer program for parallel-line bioassay, using OAF or parathyroid hormone (PTH [incubated with immunoglobulins from parental plasmacytoma cells]) as reference standards.

Immunization of animals. OAF was isolated from human tonsil cells stimulated with phytohemagglutinin as described previously (5). The purified fractions were subjected to electrophoresis in polyacrylamide gels at pH 8.5, in which the active fraction of OAF migrates at $R_f = 0.6$ (5). This region of the gel was cut out and was homogenized in 150 mM NaCl containing 20 mM phosphate buffer, pH 7.4. The homogenates were ≅50% (vol/vol) in polyacrylamide. This homogenate was injected subcutaneously into BALB/c mice on a weekly basis for 3 wk, with the equivalent of 10 μg of OAF/injection. The animals were given a final injection 3 d before sacrifice. Spleen cell suspensions were prepared by teasing in MEM, yielding ≅10^8 cells/spleen.

Hybridization of cells. The mouse plasmacytoma cell line used for hybridization was 45.6TG1.70ua3, a variant (11) of the MPC-11 cell line that lacks hypoxanthine phosphoribosyl transferase and dies in culture medium containing aminopterin (HAT medium: 12). This cell line was obtained through the Cell Distribution Center of the Salk Institute, La Jolla, Calif. The cells were cultured at 0.3–1.5 × 10^6 cells/ml in MEM supplemented with 20% fetal bovine serum (Grand Island Biological Co., Grand Island, N. Y.), glutamine, and 2-mercaptoethanol. Periodically, the cells were exposed for several days to 10 μM 6-thioguanine to destroy any cells that were positive for hypoxanthine phosphoribosyl transferase. To perform fusion of cells, both the spleen cells and the plasmacytoma cells were washed free of serum by two washes with serum-free MEM. The two cell types were then mixed, 10^7 plasmacytoma cells to 10^8 spleen cells, and were centrifuged at 250 *g* to form a mixed pellet. The pellet was resuspended in 0.8 ml of 50% polyethylene glycol 1,000 in serum-free MEM. The cells were then centrifuged at 500 *g* for 3 min, and the mixed pellet was allowed to stand at room temperature for a further 5 min without disturbance. The polyethylene glycol 1,000 was then diluted to 1% by careful additions of MEM without serum, and the cells were centrifuged again for 5 min at 250 *g*. The cells were resuspended in MEM with 20% fetal bovine serum and were distributed to wells of multiwell culture dishes. Selection with HAT medium was carried out as described by Galfre et al. (6). After ≅4 wk in culture, growth of HAT-resistant colonies was apparent, with between 1 and 20 colonies/well, estimated by inspection of the dishes at various times during selection. The supernates designated as AB-9, AB-14, etc., in Table I are from this stage of selection. Culture supernates were collected and tested for antibody activity as described above. Subsequently, cells were cloned from wells showing anti-OAF activity, by means of limiting dilution in microtiter culture dishes. Cell suspensions used for cloning were suspended in conditioned culture medium (sterilized by filtration) that had been obtained from 45.6TG1.70ua3 cells. The average density of plating was 1 cell/well; after 2 d in culture, each well was inspected under the phase microscope and only wells containing a single colony were preserved. These clones were allowed to multiply a further 2 wk, and those that had grown to sufficient density were transferred to larger culture vessels. The medium from each clone was tested for anti-OAF activity. The supernates

TABLE I

Production of Anti-OAF Activity by Hybrid Cells

Culture No.	Dilution of immunoglobulins relative to initial culture medium	Percentage of activity recovered	
		OAF	PTH
Mixed cultures			
AB-9	1:100	23*	99
	1:1,000	89	103
AB-14	1:1,000	21*	97
	1:10,000	5*	103
	1:100,000	38*	95
AC-15	1:1,000	8*	93
	1:10,000	27*	100
	1:100,000	96	102
AC-24	1:100	18*	101
	1:1,000	47*	95
	1:10,000	88	98
AC-33	1:100	29*	102
	1:1,000	79*	94
Clones from original mixed cultures			
AB-14-12	1:1,000	18*	97
	1:10,000	13*	94
	1:100,000	53*	98
AB-14-83	1:1,000	12*	99
	1:10,000	24*	105
	1:100,000	83	96
AC-15-2	1:1,000	11*	98
	1:10,000	5*	96
	1:100,000	44*	95
AC-15-36	1:1,000	8*	99
	1:10,000	10*	103
	1:100,000	64*	100

Recovery of activity was based on parallel-line bioassays of cAMP elevation in cultured mouse bone by OAF or PTH incubated with antibody, vs. OAF or PTH incubated with immunoglobulins from parental plasmacytoma cells. Each assay contained at least three doses of OAF or PTH, four bones per dose. SE of estimate are omitted for clarity; these did not exceed 10% for any tabulated value.

* Significant ($P < 0.05$) inhibition.

designated as AB-14-12, etc., in Table I were obtained from these clones.

RESULTS

Two different assays were used to measure the biological activity remaining in samples of purified OAF after incubation with hybridoma supernates: bone calcium resorption (10) and elevation of cAMP (9) in cultured mouse bones. We have shown (9) that the purified OAF fraction used in these studies stimulates both of these activities in bone at equivalent doses. Moreover, the single 9,000-dalton peptide in the fraction (5) corresponds to both the bone-resorbing and cAMP-elevating activities of the fraction in several chromatographic, electrophoretic, and ultrafiltration systems (4, 5, 10, 13, 14). Therefore, neutralization of either activity by immunogobulins isolated from hybridoma supernates was treated as evidence for specific blocking of OAF activity. Because of its relative simplicity and adaptability to larger numbers of samples, the cAMP elevation assay was used as a routine screening procedure (Table I). However, antibodies from positive clones were also tested for their activity in blocking bone resorption by OAF (Table II). In all cases, the results obtained for elevation of cAMP were duplicated by results for bone resorption.

In the cell fusions covered by the experiments in Tables I and II, a total of 144 wells (initially containing 10^5 cells/well) were established. Of these, ≅90% ultimately developed HAT-resistant colonies. There were from 1 to 20 or more separate colonies of hybrid cells in each of these wells. Of the wells containing hybrids, 15 were found to express anti-OAF activity in the isolated immunoglobulin fractions, with titers for minimal blocking activity ranging between 1:10 and 1:100,000 relative to the initial culture medium. Data from the most active five wells are recorded in the upper section of Table I. Individual clones of verified single-cell origin were isolated from wells AB-14 and AC-15 and were tested for anti-OAF activity. Of these clones, ≅15% were found to produce antibodies of the desired specificity; data from the best four of these clones are

TABLE II

Effects of Anti-OAF Hybridoma Supernates on Bone Resorption

Group	Bone-resorbing agent	Addition	Dilution of addition	Calcium release treated/control
	ng/ml			
1	None	Parental IgG	1:100	1.03±0.14
			1:1,000	1.01±0.08
2	OAF, 3	Parental IgG	1:100	3.44±0.33
			1:1,000	3.28±0.24
3	OAF, 3	AC-15-2	1:1,000	1.21±0.20*
			1:10,000	1.10±0.16*
			1:100,000	1.84±0.26*
4	OAF, 3	AB-14-12	1:1,000	0.95±0.13*
			1:10,000	1.08±0.13*
			1:100,000	1.37±0.21*
5	OAF, 3	AB-14-12 (anti-IgG treated)	1:100	3.16±0.25
6	OAF, 3	AB-14-12 (protein A treated)	1:100	3.21±0.51
7	PTH, 30	Parental IgG	1:100	3.13±0.36
8	PTH, 30	AB-14-12	1:100	3.22±0.24
			1:1,000	3.10±0.40

The indicated agents in MEM were incubated at 4°C overnight with the indicated dilutions of culture supernates. "Parental IgG" refers to immunoglobulins isolated by DEAE-cellulose adsorption (see Methods) from parental 45.6TG1.70ua3 cell culture supernates. The incubated samples were then used to culture mouse cranial bones for 72 h, at the end of which the treated/control ratios for calcium release were calculated (10), using as controls bones incubated with MEM only (groups 1–2) or bones incubated with 1:1,000 parental IgG only (groups 3–8). For groups 5 and 6, the hybridoma supernate was depleted of IgG by precipitation with anti-mouse IgG serum or with protein A before incubation. Treated/control ratios are means±SEM for four bones per point.

* Significant ($P < 0.05$) inhibition of OAF bone-resorbing activity.

TABLE III
Chromosome Counts of Hybrid Clones with Anti-OAF Activity

Clone No.	Modal chromosome number	Range (n = 20)
AB-14-12	98.6	92–104
AB-14-23	96.4	90–98
AB-14-83	92.2	88–96
AB-15-2	100.3	96–106
AB-15-36	94.5	90–96
AB-15-44	102.2	98–106

Hypotonic swelling and lysis of the metaphases were carried out as described by Klinger (15), with direct staining of the spreads using Giemsa. Counts were performed on photographs of the spreads taken under oil immersion at ×1,000.

shown in the lower section of Table I. Comparative data on the effect of these antibodies on bone resorption are presented in Table II. The resulting clones from each of these positive cultures were shown to be hybrids by chromosome counts in metaphase spreads (15), as shown in Table III. The modal chromosome number in each case was near 100, which is near the sum of the parental plasmacytoma line (modal chromosome number = 62; 11) and the normal mouse cell (i.e., 40).

The OAF-blocking activity of the hybridoma supernates was caused by immunoglobulins, as shown by the lack of adsorption of the activity to DEAE-cellulose and by the ability of either protein A (16) or anti-mouse immunoglobulin (Ig)G to abolish the activity in supernates treated with these agents before incubation with OAF (Table II). Immunoglobulins isolated from the parental plasmacytoma cell line (which secretes an IgG2b with no known antigenic specificities; 11) had no effect on OAF activity at any dilutions tested (Table II) and were therefore used in control media for all experiments. The specificity of the hybridoma antibodies against OAF was shown by their lack of effect of either bone resorption (Table II) or cAMP elevation (Table I) induced by PTH. Additional studies (data not shown) indicated that anti-OAF antibodies also had no effects on the activities of other bone-active agents including prostaglandins, vitamin D metabolites, or calcitonin.

DISCUSSION

These data demonstrate that highly sensitive and specific antibodies against OAF can be produced in useful titers by means of somatic cell hybridization followed by selection of hybridoma clones with the desired characteristics. The antibodies produced by these hybrid clones will now be screened for their suitability in design of immunoassays and immunocytochemical tests for OAF in clinical samples. Beyond these findings, however, these results validate the concept that the hybridoma production technique opens new courses of research on antigens that are either weakly immunogenic or are available in very small quantities. Antigens (such as OAF) that could not previously be used successfully to raise antisera in vivo may now be used to produce hybridomas if they are capable of activating even a very small fraction of the lymphocyte population to produce specific antibodies. The key to isolating hybridomas against such weak antigens appears to be the availability of a sensitive, specific, and reasonably rapid screening procedure, which will allow the examination of large numbers of hybridoma clones for the desired antibodies. Such assays are available in the cases of most lymphokines, hormones, and growth factors. The availability of monoclonal antibodies should be of considerable benefit in experiments on the sources, purification, and roles in disease of these factors.

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