IDENTIFICATION OF A T CELL HYBRIDOMA
THAT PRODUCES LARGE QUANTITIES OF
MACROPHAGE-ACTIVATING FACTOR*

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It is now well established that, under certain conditions, the macrophage can display effector cell function toward a variety of neoplastic cells (1–5). This process is facilitated by a series of reactions that alter the functional state of the macrophage to produce an activated mononuclear phagocyte (6–8). Current evidence indicates that activation is initiated and/or augmented by a nonspecific T cell product denoted macrophage-activating factor (MAF) (9–13).[1] MAF has been shown to develop in vitro after the interaction of immune T lymphocytes with antigen-pulsed macrophages or mitogen stimulation of normal splenic cell suspensions and is thought to alter a number of functional and biochemical properties of macrophages (reviewed in 14).

The biochemical identity of MAF, and therefore the elucidation of its mechanism of action on macrophage populations, have remained ill-defined. In part, this has been due to the limited amounts of MAF produced under conventional conditions, amounts insufficient to achieve a full biochemical purification of this factor. In addition, because conventional culture supernatants of activated T cells normally contain a heterogeneous mixture of lymphokines that can affect macrophage function such as immune interferon (IFN$_\gamma$) and migration-inhibition factor, uncertainty has arisen as to the chemical identity of MAF. Herein, MAF will be defined by its function in inducing macrophage cytocidal activity toward neoplastic cells.

To circumvent this problem, we initiated a series of experiments to test several existing murine T cell hybridomas for their ability to produce MAF. These cell lines had been constructed by fusion of alloantigen-activated T cell blasts with the BW5147 T lymphoma cell line and were previously found to produce other lymphokines, including allogenic effect factor (15) and interleukin 2 (IL-2) (16). The present study documents the identification of a T cell hybridoma that secretes large quantities of a biologically active factor that is functionally and biochemically identical to MAF produced by conventional technique. The identification of this clone should prove to be invaluable in the ultimate purification of MAF.

Materials and Methods

Media, Supplements, and Buffers. All media, supplements, and buffers used in these experiments were prepared from endotoxin-free stocks and were determined to be free of endotoxin using the Limulus Amebocyte Lysate assay (Sigma Chemical Co., St. Louis, MO). To destroy endotoxin that might have potentially been adherent to glass, all autoclaved glassware was baked for 3 h at 180°C. RPMI 1640 and minimal essential medium (MEM) were prepared from powdered medium (Flow Laboratories, Rockville, MD) using USP sterile water (Travenol Laboratories, Inc., Deerfield, IL). Liquid Dulbecco's modified Eagle medium containing 4,500 mg glucose/liter (DME) was purchased from Irvine Scientific (Santa Ana, CA). Aseptically drawn fetal calf serum (FCS) (Rehatuin F.S.) was obtained from Reheis Chemical Co., Phoenix, AZ and heat inactivated (1 h, 56°C) before use. Other media supplements included: injectable penicillin G and streptomycin sulfate (Eli Lilly and Co., Indianapolis, IN), injectable sodium bicarbonate and sodium heparin (Gibco Invenex Div., Lawrence, MA), 1 M Hepes buffer solution, versene, sodium pyruvate, and L-glutamine (M. A. Bioproducts, Walkersville, Md.), 10 mM MEM nonessential amino acids, and Trypsin-EDTA (1X) (Gibco Laboratories, Grand Island Biological Co. Grand Island, NY), gentamycin (Schering Corp., Kenilworth, NJ), Indomethacin (Sigma Chemical Co.), and concanavalin A (Con A) (Miles-Yeda, Ltd., Rehovot, Israel).

Mice. C3H/St and (BALB/c × DBA/2) F_1 hybrid mice were obtained from the breeding colony at the Research Institute of Scripps Clinic. A/St mice were obtained from West Seneca Laboratories (West Seneca, NY).

Production of Conventional MAF Supernatant. Spleens of 25 A/St mice were removed aseptically, and single-cell suspensions were prepared by passage through a Cellector Tissue Sieve (Bellco Glass, Inc., Vineland, NJ). The yield of cells was 1.2×10^8 cells per spleen and the isolated cells exhibited a viability of 80%. Cells were washed and suspended to a concentration of $5 \times 10^6/ml$ in RPMI 1640 containing 5% FCS, penicillin (50 U/ml), streptomycin (50 µg/ml), L-glutamine (2 mM), Hepes buffer solution (5 mM), 5×10^{-5} M 2-mercaptoethanol (Calbiochem-Behring Corp., San Diego, CA), and 5 µg/ml Con A. The cell suspension was incubated in T150 tissue culture flasks (Costar, Data Packaging, Cambridge, MA) (50 ml/flask) for 24 h at 37°C in a humidified, 5% CO_2 atmosphere. After incubation, the cells were removed by centrifugation at 200 g for 15 min and the supernatant filtered through a 0.2-µm sterilization filter unit (Nalge Co., Nalgene Labware Div., Rochester, NY). The resulting supernatant was stored frozen at −20°C and will heretofore be referred to as conventional MAF.

Construction and Cloning of Murine T Cell Hybridomas. T cell hybridomas were constructed by fusion of alloactivated T cell blasts with the BW5147 T lymphoma line as described previously (15). Hybridomas were grown in DME containing 10% FCS, penicillin (50 U/ml), streptomycin (50 µg/ml), L-glutamine (2 mM), and sodium pyruvate (1 mM) (complete DME) in a humidified atmosphere containing 10% CO_2. Hybridomas were cloned and subcloned as described (15, 16) by limiting dilution in 96-well flat-bottomed tissue culture plates (Costar). Each well contained an average of 0.5 hybridoma cells and 2×10^6 irradiated BALB/c thymocytes.

Growth and Stimulation of T Cell Hybridoma Clones. Selected T cell hybridoma clones were maintained in complete DME containing 5% FCS. The cells, which are partially adherent, were grown until the density of nonadherent cells reached 1×10^6 viable cells/ml. 10% of the culture was transferred, and the remaining 90% of the culture was stimulated with Con A (10 µg/ml final concentration). After incubation for 24 h at 37°C, the supernatant was harvested. Cells were removed by centrifugation and the supernatant was sterile filtered as described above.

Macrophages. C3H/St mice were injected intraperitoneally with 1.5 ml of sterile proteose peptone (10% wt/vol, Difco Laboratories, Detroit, MI) 3 d before harvest. Cells were harvested by peritoneal lavage using RPMI medium containing 2% FCS, antibiotics, L-glutamine, 10 mM Hepes buffer, and sodium heparin (10 U/ml). Cells were washed at 4°C in the above buffer lacking heparin (assay washing buffer) and resuspended to $1 \times 10^6/ml$ in RPMI medium containing 5% FCS, penicillin (50 U/ml), streptomycin (50 µg/ml), L-glutamine (2 mM), sodium bicarbonate (0.075% wt/vol), and sodium pyruvate (1 mM) (complete RPMI). The yield of cells averaged 6×10^6 peritoneal exudate cells per mouse and cell viabilities were

>95%. Approximately 80% of the final cell suspension represented macrophages based on histochemical, functional, and morphological criteria. For use in the ^{51}Cr release tumoricidal assay for MAF, 200 μl of the suspension (2 $\times$ 10^5 peritoneal exudate cells) was added into 6.4-mm Diam, flat-bottomed wells in 96-well tissue culture plates. The plates were centrifuged for 10 min at 200 g and then transferred to a 37°C incubator for 2 h to allow for cell adherence. After the adherence step, the wells were subjected to vigorous washing to remove nonadherent cells. Plates were used in the assay immediately after this last step.

Target Cells. The murine mastocytoma cell line P815 was used as a target in the MAF-dependent tumoricidal assay. P815 cells were maintained in culture as well as by weekly in vivo transfer through (BALB/c $\times$ DBA/2) F$_1$ mice. 2 $\times$ 10^7 P815 cells in 500 μl complete RPMI were labeled by incubation for 1 h at 37°C with 200 μCi of sterile ^{51}Cr (Na$_2$CrO$_4$; New England Nuclear, Boston, MA). Cells were washed, suspended in medium to 2 $\times$ 10^6 cells/ml, and incubated an additional 1 h at 37°C to eliminate the rapid phase of ^{51}Cr release. Cells were pelleted and washed once more with 50 ml of complete RPMI. ^{51}Cr-labeled P815 cells were then suspended to 1.5 $\times$ 10^5/ml in complete RPMI for use in the assay.

^{51}Cr Release Tumoricidal Assay for MAF. The ^{51}Cr release tumoricidal assay used in these studies will be reported in detail elsewhere, but is basically similar to that described by Pace and Russell (17). Briefly, 2 $\times$ 10^5 peptone-elicited macrophages adherent in 96-well tissue culture plates (see above) were incubated, in triplicate, with 200 μl of serial dilutions of MAF-containing supernatants in complete RPMI medium. Because MAF represents only one of the two activation signals required to induce macrophage-dependent tumoricidal activity, the wells also contained 1 $\times$ 10^6 heat-killed *Listeria monocytogenes* (HKLM) organisms, which were shown previously to act as a second or triggering signal. Reaction mixtures also contained 1 $\times$ 10^{-6} M indomethacin to prevent inhibition of macrophage tumoricidal activity by prostaglandins. Control wells, which were used to determine spontaneous release, contained the macrophage monolayers, HKLM, and indomethacin, but not MAF. After incubation for 4 h at 37°C in a 5% CO$_2$ atmosphere, the macrophage monolayer was washed three times in complete RPMI medium containing 1 $\times$ 10^{-6} M indomethacin. 3 $\times$ 10^4 ^{51}Cr-labeled P815 cells in 200 μl of the above medium were added to the wells (effector/target cell ratio = 6.7:1), and cell-cell contact was initiated by centrifugation of the reaction plate for 10 min at 200 g. Cultures were incubated for 16 h at 37°C in a humidified 5% CO$_2$ atmosphere. At the completion of incubation, plates were centrifuged for 10 min at 200 g and 100-μl samples removed from each well. The radioactivity content of each sample was assessed in an automatic gamma counter (Micromedic Systems, Horsham, PA), and the percent specific ^{51}Cr release was determined using the formula: percent specific release = 100 $\times$ ([experimental release − spontaneous release]/[total release − spontaneous release]). Total releasable counts were determined in quadruplicate by subjecting labeled targets to two cycles of freezing and thawing. Spontaneous release from ^{51}Cr-labeled P815 averaged 1–2%/h. 1 U of MAF is defined as that amount which produces 50% maximal ^{51}Cr release in this assay.

Interferon Assay. Interferon was identified by its ability to protect murine L cells (clone 929) from infection by vesicular stomatitus virus (VSV) using the cytopathic effect assay (18). Purified VSV was prepared by published methods (19) and was a gift from Dr. D. Beebe of this institute. Serial twofold dilutions of interferon were mixed, in triplicate, with 3 $\times$ 10^4 mouse L cells in flat-bottomed 96-well tissue culture plates. The total reaction volume was 200 μl and the medium was MEM containing 10% FCS, gentamycin (50 μg/ml), L-glutamine (2 mM), sodium bicarbonate (0.075% wt/vol), sodium pyruvate (1 mM), and MEM nonessential amino acids (0.1 mM). After incubation for 18 h at 37°C, L cell monolayers were washed and then infected with 200 μl of diluted, purified VSV. Plates were read when control wells, which received VSV but not IFN, showed a complete cytopathic effect (36–48 h postinfection). 1 U of IFN is defined as that amount providing the L cell monolayers with 50% protection from the cytopathic effects of the virus.

IL-2 Activity. The assay for IL-2 was based upon the ability of this lymphokine to support the growth of the IL-2-dependent T cell line CTLL-2 in a short-term (24 h) assay, using 1 $\times$ 10^4 CTLL-2 cells and serial dilution of test supernatants, as described (16).

Gel Filtration Chromatography of Hybridoma MAF. 80 ml of clone 24/G1 MAF was concentrated to 5 ml and applied to a 2.5 $\times$ 100-cm column of Sephadex G100 (Pharmacia Fine Chemicals,

Div. of Pharmacia Inc., Piscataway, NJ). The column was eluted at 25 ml/h at 4°C with a buffer that contained NaCl (0.25 M), Hepes buffer (0.02 M), and α-methyl-D-mannoside (0.1 M). Samples of the fractions were diluted in medium and sterile filtered before testing in the MAF assay.

Chromatofocusing. The pool containing the majority of the MAF activity that had eluted from the Sephadex G100 column (pool III, fractions 37–47) was concentrated to 6 ml and dialyzed against 25 mM midazole-HCl, pH 7.4. The sample was applied to a 1.0 × 18.5-cm column of Polybuffer Exchanger PBE-94 (Pharmacia Fine Chemicals), which had been equilibrated at pH 7.4 in 25 mM imidazole-HCl buffer. The column was eluted at 4°C with 210 ml of Polybuffer 74-HCl (Pharmacia Fine Chemicals), diluted 1:8, and adjusted to pH 4.0. pH values of the eluted fractions were determined using a standard pH meter (Radiometer, Copenhagen, Netherlands) and then adjusted to neutrality by addition of 1 M Hepes buffer solution. Samples of the fractions were diluted in medium and sterile filtered before testing in the MAF assay.

Results

Demonstration of Secretion of MAF Activity by T Cell Hybridoma 24. Of four parental hybridomas tested for their ability to secrete MAF, only one (hybridoma 24) could be identified as an MAF producer (Table I). Although no MAF activity could be detected in culture supernatants from unstimulated cells, incubation of hybridoma 24 with mitogens such as Con A or phytohemagglutinin provoked the expression of MAF activity in the culture fluid. MAF activity was near maximal at a dilution of 1:100 of the stimulated culture supernatant, but disappeared at a dilution of 1:500. This level of MAF represents ~20–30% of the activity found in conventional MAF preparations (MAF 8080) produced by Con A stimulation of normal murine splenic cells. The amount of MAF released from hybridoma 24 was dependent on the mitogen concentration used. For Con A, maximal MAF release occurred using 10–20 μg/ml of the mitogen whereas 40 μg/ml was inhibitory. Stimulated culture supernatants of hybridoma 24 also displayed IFN antiviral activity and IL-2 activity.

Isolation of Clone 24/G1. Cloning of parental hybridoma 24 at an average density of 0.5 cells/well yielded no clones producing MAF constitutively. Fig. 1 demonstrates that 24 clones produced MAF when stimulated with 10 μg/ml Con A. At least seven of the clones (A6, C7, D10, E3, G1, G5, and H4) produced more MAF activity than the conventional MAF standard (last column on far right). An additional nine clones, which were found to produce >200 U MAF/ml during the original screening, have not been characterized by titration and are therefore indicated by the broken bars

TABLE I

Demonstration of MAF Activity Secreted From T Cell Hybridoma 24 Upon Stimulation With Con A

Dilution of MAF source	Percent specific P815 lysis			
	Hyb 24/Con A*	Hyb 24‡	Con A§	MAF 8080‖
1:20	51.4	3.9	0.0	49.8
1:100	43.7	N.D.	N.D.	51.1
1:500	4.2	N.D.	N.D.	29.1
1:2500	0.0	N.D.	N.D.	6.8

* Culture of hybridoma 24 incubated 18 h with 10 μg/ml Con A.
‡ Unstimulated control of hybridoma 24.
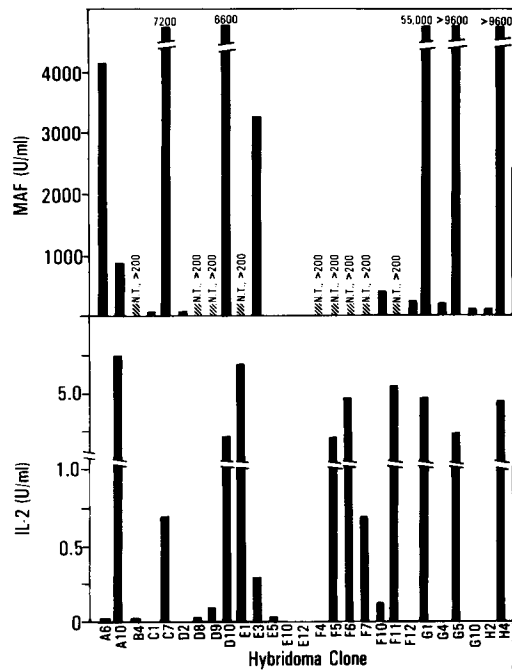§ Con at 10 μg/ml incorporated in the lysis assay.
‖ Splenic MAF.

Fig. 1.   MAF and IL-2 production by clones of T cell hybridoma 24. 5-ml cultures of 27 T cell clones were stimulated for 24 h with 10 μg/ml Con A in DME containing 5% FCS. None of the clones produced MAF constitutively, but 15 of 27 produced IL-2 constitutively. MAF and IL-2 activities of a conventional MAF standard prepared by Con A stimulation of a normal murine splenic cell culture are shown by the vertical bars on the far right.

TABLE II

Independent Production of MAF and IL-2 by Hybridoma 24 Clones

Clone	Lymphokine activity (percent of standard)*	
	MAF	IL-2
A6	175	2
A10	33	675
E3	137	50
G1	2,500	440
G4	4	0
G5	800	208
H4	800	440

* Standard was Con A-stimulated normal spleen cell culture supernatant.

labeled: N.T. > 200.

Fig. 1 also quantitates the production of IL-2 by the clones. Unlike MAF, IL-2 was produced as a constitutive product by 15 out of 27 clones, although only in low concentrations. Upon stimulation with Con A, 18 of the clones responded with IL-2 secretion. Ten clones (A10, C7, D10, E1, F5, F6, F11, G1, G5, and H4) produced more IL-2 than did normal mouse splenic cells stimulated under the same conditions. Table II demonstrates that expression of MAF and IL-2 activities in stimulated supernatants of the various clones varied independently of one another. Clone 24/A6

produced high levels of MAF but low amounts of IL-2, whereas clone 24/A10 produced high concentrations of IL-2 but only minimal levels of MAF. Clones 24/G1, 24/G5, and 24/H4 secreted high amounts of both lymphokines, but the quantitative levels of the two activities varied independently of one another.

When cytotoxicity titrations were performed (Fig. 2), the supernatant from clone 24/G1 was found to contain 55,000 U of MAF/ml and was 23 times greater than the conventional MAF standard (2,400 U/ml). To rule out the possibility that the hybridoma MAF contained an enhancing activity or that the conventional MAF contained a MAF inhibitor, hybridoma MAF and conventional MAF were mixed at varying ratios and then titered in the cytotoxicity assay. Table III shows that the above possibilities are unlikely because the MAF activity of the mixtures closely approximated calculated theoretical values.

Comparison of Heat and pH Sensitivities of Hybridoma MAF and Conventional MAF.   Fig. 3 shows that the hybridoma MAF was stable to incubation for 1 h at 4°C, 37°C, and 56°C, but was inactivated when held at 65°C or 80°C for 60 min. Exposure of the preparation to 100°C for 20 min also abrogated the MAF activity. This pattern of heat sensitivity is identical to that of conventional MAF, but differs with results reported elsewhere (20) that MAF is inactivated after 1 h at 56°C. This difference,
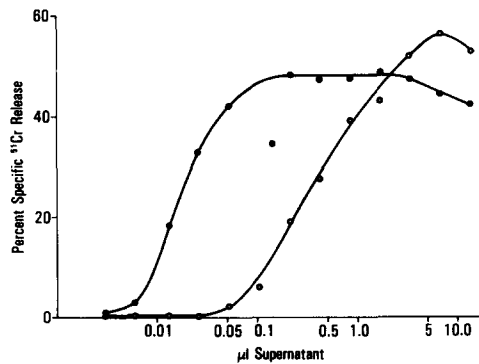

Fig. 2.   MAF titration of a Con A-stimulated supernatant of T cell hybridoma clone 24/G1 (●). The dose-dependent ability of the hybridoma MAF to induce tumoricidal activity in peptone-elicited macrophages is compared with the activity present in the Con A-stimulated splenic cell culture supernatant (○). The latter is the conventional MAF standard used in this study. The ^{51}Cr-release tumoricidal assay was performed as described in Materials and Methods.

TABLE III

*Mixing of Hybridoma (24/G1) and Conventional (8080) MAF Preparations:
Lack of Enhancement or Inhibition*

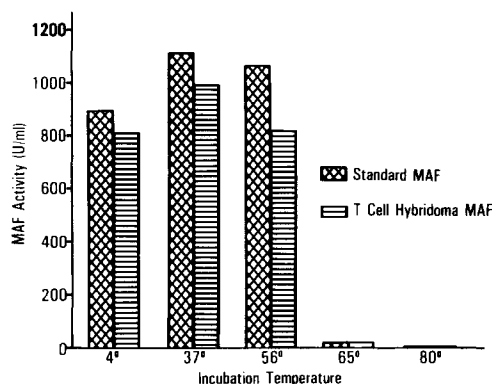| MAF ratio 24/G1:8080	MAF Activity	
	Measured	Theoretical
	U/ml	
1:0	51,200	—
0:1	3,200	—
3:1	35,800	39,200
1:1	25,600	27,200
1:3	16,000	15,200

Fig. 3. Comparison of heat sensitivities of T cell hybridoma MAF and conventional MAF preparations. 5-ml samples of each MAF preparation in medium containing 5% FCS were held at the designated temperatures for 1 h and then titrated for MAF activity in the ^{51}Cr-release tumoricidal assay.
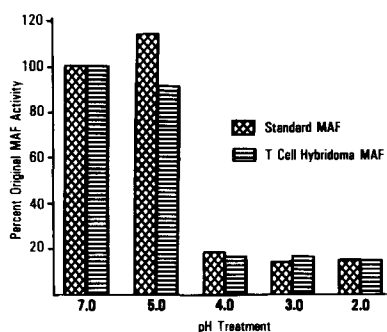

Fig. 4. Comparison of pH sensitivities of T cell hybridoma MAF and conventional MAF preparations. 5-ml samples of each MAF-containing supernatant were subjected to dialysis for 18 h at 4°C against 1 liter of physiological saline buffered to the designated pH values. Samples were then back dialyzed into 0.1 M phosphate buffer pH 7.4 for 4 h and then dialyzed into phosphate-buffered saline. Treated samples were titrated for MAF activity and the amount of activity remaining compared to the untreated controls.

however, is caused by the presence of FCS in our MAF preparations, as stimulation of hybridoma 24 with Con A in serum-free medium results in the production of MAF that is labile to 56°C (64% activity lost after 1 h).

Fig. 4 demonstrates that hybridoma MAF and conventional MAF share identical pH sensitivities. Treatment of both preparations at or below pH 4.0 resulted in the loss of 80% of the MAF activity. No loss in activity was found upon treatment at pH 7.0 or 5.0. Identical results were obtained when the pH of the lymphokine solutions was adjusted in the test tube by titration with HCl and held constant for 6 h at 4°C before neutralizing them with NaOH.

Demonstation of a Requirement for a Second Signal in Induction of Macrophage Cytocidal Activity by Hybridoma and Conventional MAF. It has previously been found that the in vitro induction of macrophage tumoricidal activity requires the participation of a second signal that is distinct from MAF (17, 21–23). A variety of substances, including endotoxin, have been found to act as second signals (reviewed in 22). In our own experiments (23), we have found that either heat-killed HKLM or endotoxin can

enhance MAF activity 50-fold in the tumoricidal assay. Table IV demonstrates that similar enhancement of MAF activity by a second signal is observed for both hybridoma MAF (42-fold) and conventional MAF (50-fold).

Gel Filtration Chromatography of Hybridoma MAF on Sephadex G100. Conventional MAF has been shown to have a molecular weight of 40,000–60,000 when subjected to molecular sieve chromatography (20, 23, 24). A 16-fold concentrated sample of 24/G1 MAF was applied to a column of Sephadex G100 to determine the molecular

TABLE IV

Demonstration of a Requirement for a Second Signal in the Induction of Macrophage Tumoricidal Activity by Conventional MAF and Hybridoma MAF

MAF preparation	MAF activity	
	Plus HKLM*	Minus HKLM
	U/ml	
Conventional MAF (8080)	1,750	35
Hybridoma MAF (8171)	21,420	510

* HKLM used at a final concentration of 5 × 10⁶/ml as a source of second signal.
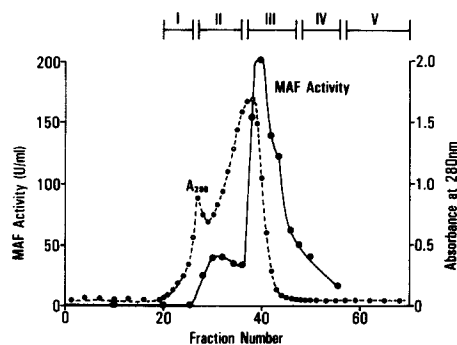

Fig. 5.   Gel filtration chromatography of T cell hybridoma MAF on Sephadex G100. 80 ml of hybridoma MAF was concentrated to 5 ml by ultrafiltration and applied to a 2.5 × 100-cm column of Sephadex G100 equilibrated in 0.02 M Hepes buffer containing 0.24 M NaCl and 0.1 M α-D-methyl mannoside. Elution was performed at 4°C at a flow rate of 25 ml/h. 5-ml fractions were collected. Fractions were pooled as designated by the vertical vars and roman numerals.

TABLE V

Recoveries of MAF Activity From Sephadex G100 Column

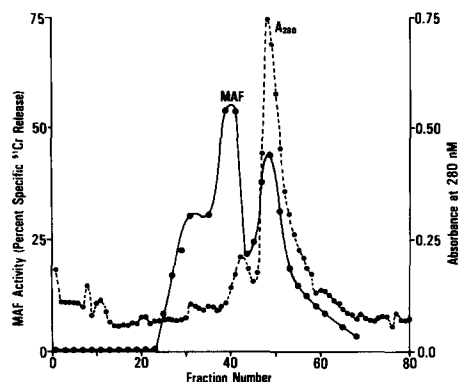| Column pool	MAF activity	
	Total units	Percent recovery
I	4,110	0.4
II	140,880	13.8
III	413,440	40.5
IV	21,120	2.1
V	8,220	0.8
I–V (Calculated)	587,770	57.6
I–V (Measured)	479,040	47.0
Unfractionated	1,020,000	—

Fig. 6. Chromatofocusing of hybridoma MAF following gel filtration. Pool III from Fig. 5 was concentrated to 6 ml by ultrafiltration and dialyzed against 2 liters of 0.025 M imidazole-HCl pH 7.4. The sample was applied to a 1.0 × 18.5-cm column of Polybuffer Exchanger-94 and eluted at 4°C with 210 ml of Polybuffer 74-HCl diluted 1:8 and adjusted to pH 4.0. Fraction size was 2.5 ml.

weight of hybridoma MAF. The column was eluted with buffer that contained high salt and 0.1 M α-methyl-D-mannoside. This buffer has been found to greatly reduce adsorption of MAF activity onto carbohydrate-containing separation media; the enhanced yield of MAF in the presence of α-methyl-D-mannoside appears to reflect the ability of MAF to interact with mannose residues in a lectinlike fashion.

As shown in Fig. 5, the major amount of MAF activity eluted in a position behind bovine serum albumin and displayed a molecular weight of 55,000. This value agrees with the 50,000 mol wt determined for a conventional MAF preparation under the same conditions. The hybridoma MAF activity eluted in a slightly asymmetrical manner with an increase in activity on the trailing side of the elution peak. A minor percentage (<10%) of activity was detectable in the fractions that corresponded to the void volume of the column. Fractions delineated by the vertical bars in Fig. 5 were pooled, and the resulting pools were titrated for MAF activity. Table V indicates that pool III (fractions 37–47) contained 40% of the MAF activity that had originally been applied. To test for the possibility that the gel filtration step might have separated two distinct activities, aliquots of pools I–V were combined and titrated for MAF activity. Table V indicates that this possibility is unlikely, as the measured MAF content of the combined pools (479,040 U) is in agreement with the calculated MAF content of the five individual pools (587,770 U). In fact, the measured recovery (47%) is actually slightly lower than the calculated recovery (57.6%).

Chromatofocusing of Hybridoma MAF. The major activity-containing pool III of the hybridoma MAF was subjected to chromatofocusing. Fig. 6 demonstrates that MAF activity eluted as at least two distinct species. The major peak eluted at pH 5.45 and contained 60% of the activity. Very little protein was found in this region of the elution profile. A leading edge of activity was observed for this predominant activity peak. A second distinct peak eluted at pH 4.90 and was found to coelute with the major protein peak, which was subsequently found to be BSA derived from the FCS in the culture medium.

Discussion

This report constitutes the first description of a T cell hybridoma that secretes a lymphokine that induces tumoricidal activity in macrophages; as designed herein,

this lymphokine is MAF.

MAF has been reported to elicit or alter a variety of biological responses from macrophages, including increases in endocytic, biosynthetic, secretory, and effector cell functions as well as changes in membrane physiology and composition (reviewed in 14). The current, generally held concept is that two signals are required to generate tumoricidal activity in macrophages (17, 21–23). MAF is the first signal which primes the macrophage for "triggering" by a second signal (22). Second signals constitute a heterogeneous group of substances which can either be host-derived or be of nonhost origin.

The results presented in this study demonstrate that a murine T cell hybridoma that secretes MAF has been obtained. Although not produced constitutively, mitogen stimulation of the hybridoma cell culture induced production of high levels of MAF; indeed, in quantities considerably greater than could be detected in conventional MAF preparations generated by Con A stimulation of normal murine spleen cell cultures. The activity secreted by the T cell hybridoma was shown to be identical with conventional MAF by a variety of functional and biochemical criteria. Both preparations required an additional component or "second signal" to induce tumoricidal activity in peptone-elicited murine macrophages and exhibited identical heat and pH denaturation profiles, and the MAF activities from both supernatants displayed identical apparent molecular weights of 50–55,000 as measured by gel permeation chromatography on Sephadex G100.

The characteristics of molecular weight, inactivation after treatment at pH 4.0 or 65°C, and the requirement for a second signal to induce macrophage tumoricidal activity displayed by our preparations of hybridoma-derived or conventional MAF are in agreement with those reported for conventional MAF by others (20, 24). The observation that MAF activity in our culture supernatants was stable to incubation for 1 h at 56°C appeared to be at variance with at least one other report on conventional MAF (20). However, these disparate results were reconciled by the finding that the FCS used in our culture supernatants had a protective effect on the heat denaturation of MAF.

The higher amount of MAF activity displayed by the T cell hybridoma supernatant when compared with the conventional MAF preparation represents an actual increase in concentration or specific activity of the hybridoma-secreted lymphokine. This conclusion was supported by the mixing experiments presented in Tables III and V. These results indicate that conventional MAF preparations do not contain inhibitors that could depress the expression of MAF cytocidal-inducing activity. When subjected to gel filtration chromatography, the hybridoma MAF eluted as a single activity peak and was recovered in high yield. Mixing aliquots of all the fractions from the column did not increase the recovery of MAF activity. These results indicate that no additional factor was present in the hybridoma MAF preparation that was distinct from MAF on the basis of molecular weight, but that was required for MAF-dependent induction of cytocidal activity.

The cloning experiments performed in the study indicated that distinct T cell hybridoma clones can produce different repertoires of individual lymphokines. The differences observed in lymphokine levels could be either qualitative or quantitative. Whereas supernatants of some clones contained high levels of MAF, supernatants of other clones displayed only limited levels of the activity. Several clones which

produced MAF in high concentration produced only limited quantities of other lymphokines (such as IL-2) and vice-versa. Although MAF was not secreted as a constitutive product by any of the clones, IL-2 secretion by several clones could be detected in the absence of mitogen stimulation.

By comparing the lymphokine titers in culture supernatants of different clones, it should be possible to distinguish between certain lymphokine activities. For instance, from past studies it has remained unclear whether MAF and IFN are identical or distinct. Certain types of IFN (α and β) have been shown to activate macrophage tumoricidal activity (25, 26). In addition, IFNγ displays a molecular weight, pH sensitivity, and heat stability (27) similar to MAF. Thus it would be of considerable interest if the two activities could be differentiated using the T cell hybridoma clones. In fact, in the present study, four clones were initially identified that produced high levels of MAF activity and only limited amounts of IFN antiviral activity. One of these clones (24/G1) produced no detectable amounts of IFN activity despite the expression of a very high level of MAF activity. However, upon expansion of this clone, stimulated culture supernatants were found to contain both MAF and IFN activities. Two posibilities that might explain this result are currently being explored: (a) the initial hybridoma clone may have produced both MAF and IFN but the IFN antiviral activity was masked by an unknown inhibitory factor in the hybridoma supernatant. Upon expansion of the clone, the gene for this factor was lost, thereby allowing for expression of the IFN antiviral activity. (b) Expression of MAF and IFN activities may differ kinetically, due either to different rates of biosynthesis or secretion or to different rates of degradation or interconvertability.

To date, only one other report (28) exists documenting the production by a murine T cell hybridoma of lymphokines that directly effect macrophage function. This study described supernatants from a T cell hybridoma that produced inhibition of macrophage migration and induced C2 and elastase synthesis or release by murine macrophages. The levels of activity measured in the hybridoma supernatant were comparable to or slightly elevated above (two- to threefold) the activity levels observed in supernatants from Con A-stimulated spleen cell cultures. One particular clone, which exhibited the highest titer of C2-inducing activity, was devoid of IFN antiviral activity. It is unknown whether the factor(s) that was produced by this hybridoma is the same as the MAF produced by our hybridoma.

The identification of the T cell hybridoma that produces large quantities of MAF constitutes the first step in providing the needed starting material for the biochemical purification of this lymphokine. Once purified, MAF can be characterized with respect to the biological functions it elicits and the mechnism by which these effects are produced. Hybridoma culture supernatants are currently being produced at a rate of 1–2 liters/wk. Each liter contains the equivalent of the MAF activity that can be produced by the spleen cell cultures of 4,000 mice. Work is currently underway to devise a large-scale isolation procedure for the T cell hybridoma-derived MAF.

Summary

A murine T cell hybridoma, constructed by fusion of alloantigen-activated T cells with the BW5147 T cell lymphoma, which produces a lymphokine capable of inducing tumoricidal activity in macrophages, has been identified. Lymphokine release could be detected only after mitogen stimulation of the T cell hybridoma culture. Upon

cloning of the parental hybridoma, 24 out of 27 clones produced tumoricidal-inducing activity. Seven clones produced more cytocidal-inducing activity than did conventional supernatants, generated by concanavalin A stimulation of normal murine spleen cell cultures, whch contained macrophage-activating factor (MAF). The supernatant of hybridoma clone 24/G1 was 25 times more active than conventional MAF preparations. Using supernatants from a variety of clones, the levels of macrophage-activating activity and interleukin 2 were found to vary independently of one another. The lymphokine produced by hybridoma clone 24/G1 appeared to be identical to conventional MAF by a variety of criteria including: (a) a requirement for a second signal for induction of tumoricidal activity in macrophages, (b) inactivation after incubation for 1 h at 65°C, and (c) loss of activity after treatment at pH 4.0 but not at pH 5.0. Like conventional MAF, the hybridoma MAF eluted as a single peak after molecular sieve chromatography on Sephadex G100 and exhibited an apparent molecular weight of 55,000. Although somewhat heterogeneous, the majority of hybridoma 24/G1 MAF displayed an isoelectric point of 5.4 as determined using the chromatofocusing technique. These results thus illustrate the usefulness of T cell hybridomas in distinguishing between various lymphokine activities and indicate that the T cell hybridoma clone 24/G1 will be of particular usefulness in achieving the biochemical purification of substantial quantities of murine MAF.

Note added in proof: While this paper was in production, another study appeared that also documented the production of MAF by a murine T cell hybridoma (Ratlif, T. L., D. L. Thomasson, R. E. McCool, and W. J. Catalona, 1982. Production of macrophage-activation factor by a T-cell hybridoma. *Cell. Immunol.* **68:**311).

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