HYBRIDOMA
Volume 6, Number 3, 1987
Mary Ann Liebert, Inc., Publishers

Preparation and Characterization of Monoclonal Antibodies Directed Against Antigenic Determinants of Recombinant Human Tumour Necrosis Factor (rTNF)

ANTHONY MEAGER, SHARAN PARTI, HELEN LEUNG, ELIZABETH PEIL, and BERNARD MAHON

Division of Immunobiology, National Institute for Biological Standards and Control, Holly Hill, Hampstead, London NW3 6RB, United Kingdom

ABSTRACT

A large number of monoclonal antibodies (McAb) binding to antigenic determinants of human tumour necrosis factor (TNF) were prepared from two fusions of mouse myeloma NSO cells with spleen cells from Balb/c mice immunized with highly purified recombinant (r)TNF. Several of these McAbs were highly neutralizing with respect to the biological activity (cytotoxicity) of TNF manifested in L-929 Cl.10 cells. Antibody competition experiments suggested the presence of at least two antigenic determinants on the rTNF molecule through which binding of McAb effects neutralization of biological activity. Some of these McAbs were shown to be suitable for the development of immuoassays to quantify rTNF.

INTRODUCTION

Carswell et al (1) identified a cytotoxin (CTX) in the sera of mice infected with Bacillus Calmette-Guerin (BCG) and subsequently treated with endotoxin which they called TNF because of its ability to induce selective tumour necrosis. It has been subsequently found that activated macrophages are the major cellular source of TNF (2) and that, in addition to its selective cytotoxicity, it has other profound biological effects (3). Several of these effects have been ascertained using highly purified rTNF derived from E.coli (4,5). The availability of TNF-specific neutralising antibodies has also aided elucidation of the various biological properties of TNF (6-10). In this study we set out to prepare anti-human TNF monoclonal antibodies (McAbs), for which, to our knowledge, there are as yet few reports on their actual preparation and characterization (11,12), in order to learn more about the antigenic structure of TNF and to prepare reagents for TNF quantification by immunoassay.

MATERIALS AND METHODS

Antibody Preparation

Recombinant human TNF (BASF Knoll AG, Ludwigshafen, FRG; $1.5x10^7$ units/mg protein) was used in all studies. Polyclonal antiserum was produced in rabbits immunised intramuscularly (im) with 100µg rTNF in Freund's complete adjuvant (FCA) and subsequently boosted at 4-6 week intervals with the same dose of rTNF in incomplete Freund's adjuvant (IFA). Serum from successive bleeds was tested in TNF-neutralisation assays (see below) and the most potent serum samples combined and purified by ammonium sulphate precipitation followed by gel filtration in Ultrogel ACA44 (LKB). Anti-rTNF McAbs were prepared by the Köhler and Milstein (13) technique following immunisation of young Balb/c mice with 50µg rTNF in FCA (day 0, im), 50µg rTNF in IFA (day 28, im) and 100µg rTNF intravenously 3 days (day 126 and 196) before fusions with NSO were carried out. Hybridoma wells were screened by solution phase immunoprecipitation tests (IPT) (14) using ^{125}I-rTNF labelled by reaction with Enzymobead reagent (Bio-Rad Laboratories, Richmond, CA, USA) to preserve biological activity of rTNF. Hybridoma cells from positive wells were cloned in semi-solid agar, anti-rTNF McAb producing clones identified by ^{125}I-rTNF IPT, recloned and subsequently expanded for cryopreservation and ascitic fluid production. Immunoglobulin subclass determination was carried out using standard procedures.

TNF Cytotoxicity Assays

L929 C1.10 cell monolayers in microtitre trays were stained after 24 h incubation in medium containing rTNF plus actinomycin D (AMD; 1µg/ml) with amido blue black (30 min; 0.05% in 0.1M sodium acetate-9% acetic acid), fixed (10 min; 0.1M sodium acetate-9% acetic acid-10% formaldehyde), dye re-extracted in 0.05M NaOH and absorbances read at 620nm in a Titertek Multiskan. Titres were interpolated from dose response curves, the end-point being defined as the median absorbance or OD_{620} on the scale of dye uptake measurements that range between those of untreated cells (negative control) and those killed with a high dose of rTNF (positive control). A laboratory reference preparation of rTNF was was included in all assays. To estimate the neutralizing capacity of rabbit polyclonal anti-rTNF and anti-rTNF McAbs, serial antibody dilutions were first incubated with rTNF, at a concentration just sufficient to kill all of the L-929 C1.10 cells, in medium containing AMD for 2 h at 37°C. These diluted antibody-rTNF mixtures were then transferred to preformed L-929 C1.10 monolayers and cytotoxicity assays processed as described previously. Antibody neutralizing titre was defined as the reciprocal of that antibody dilution giving the median OD_{620} measurement on the scale of dye uptake measurements that range between positive and negative controls.

Immunoassays to Quantify TNF

(i) imunoradiometric assay (IRMA) This was adapted from a method described by Secher (15) for the quantification of human interferon alpha (HuIFNα). Briefly, 6.5mm diameter etched polystyrene beads coated with purified rabbit polyclonal anti-rTNF or anti-rTNF McAb (100µg protein per ml in PBS) were incubated with serial dilutions of rTNF reference preparation for 4 h at 4°C, washed three times and then incubated with ^{125}I-2.33.G2 or ^{125}I 2.101.23 anti-rTNF McAb,

radioiodinated by the chloramine T method (16), for 16 h at 4°C. The beads were re-washed and counted in an LKB gamma counter.

(ii) enzyme-linked immunosorbent assay (ELISA) Purified rabbit polyclonal anti-rTNF diluted 1:30 in PBS was coated on U-bottomed polyvinyl chloride (PVC) microtitre plates (Dynatech), 50µl/well, for 2 h at 37°C. Remaining sites in wells were blocked with 2% BSA-PBS, 150µl per well, overnight at 4°C. Excess antibodies and blocking buffer were then removed and wells washed 4 times with 0.05% Tween 20 (TW20)-PBS. Following the last wash, serial dilutions of rTNF laboratory reference preparation, 50µl per well, were added and incubated at 37°C for 1 h. Wells were washed again 4 times with TW20-PBS followed by (a) addition of purified anti-rTNF McAb, 1:100 in 2% BSA-PBS, 50µl/well, and incubation for 1 h at 37°C, (b) washing with TW20-PBS and addition of biotinylated sheep anti-mouse immunoglobulin (Amersham International, UK), 1:500 in 2% BSA-PBS, 50µl/well and incubation for 1 h at 37°C, (c) washing with TW20-PBS and addition of streptavidin biotinylated horseradish peroxidase complex (Amersham International, UK), 1:500 in 2% BSA-PBS, 50µl/well and incubation for 30 min at 37°C, (d) washing with TW20-PBS (twice) and 0.1M citrate/phosphate buffer, pH 5.0, (twice) and addition of substrate, orthophenylenediamine (OPD), 1mg/ml, in 0.1M citrate/phosphate buffer, pH 5.0, containing 0.006% hydrogen peroxide (H_2O_2). Colour (yellow) was developed in the dark for 30 min at room temperature and the reaction terminated by addition of 50µl 1M H_2SO_4 to each well. Optical densities were read at 450nm in a Titertek Multiskan.

RESULTS AND DISCUSSION

Screening of two fusions by ^{125}I-rTNF IPT showed that 90 of 760 wells contained anti-rTNF McAb-secreting hybridomas. Only about 15% of these produced supernatants with significant neutralising capacity against the cytotoxicity of rTNF. Some 27 of the 90 positives were cloned, with emphasis being placed on obtaining the most potent neutralising McAbs. This process yielded 24 stable hybridoma clones, 13 of which, when grown as ascites, produced highly neutralising anti-rTNF McAbs (Table 1). The latter were of comparable potency to rabbit polyclonal anti-rTNF which titred at 24000 nu/ml. All of the anti-rTNF McAbs so far tested, whether of high or low neutralising capacity, were mouse IgG_1 subclass.

2.33.G2 and 3.101.23 anti-rTNF McAbs were purified from ascitic fluid by protein A-sepharose chromatography (17) and labelled with ^{125}I using the chloramine-T method (16). These radio-iodinated McAbs were used in antibody competition experiments (Table 1) which showed that anti-rTNF McAbs could be segregated into three broad groups (Table 1), suggesting the existence of three or more antigenic determinants or epitopes in the rTNF molecule. Highly neutralising anti-rTNF McAbs were divided between the epitope recognised by 2.33.G2 and that recognised by 3.101.23 (Table 1), whilst the majority of low and non-neutralising McAbs, which did not compete out the binding of either radio-iodinated McAb, presumably bound to one or more different epitopes. Neutralisation of rTNF cytotoxicity probably occurs because antibody-binding sterically inhibits rTNF molecules attaching to their cell surface receptors. Alternatively, that such neutralisation can occur through McAbs binding to two or more separate epitopes suggests that two or more distinct areas or sites on the rTNF molecule might be necessary for receptor binding.

L-cell cytotoxicity assays for rTNF are quite easily performed,

TABLE 1 NEUTRALISATION OF rTNF BY MONOCLONAL ANTIBODIES AND EPITOPE ANALYSIS OF rTNF†

Epitope 1 (defined by binding of ^{125}I-2.33.G2)		Epitope 2 (defined by binding of ^{125}I-3.101.23)		Other epitopes (not recognised by either ^{125}I-2.33.G2 or -3.101.23	
McAb	nu/ml	McAb	nu/ml	McAb	nu/ml
2.33.G2	24000	3.88.2	24000	2.28.C6	1500
2.178.B8	12000	3.88.34	56000	3.49.20	80
2.179.F9	24000	3.101.23	24000	3.76.22	80
3.6.10	8000	3.D1.11	12000	3.165.48	320
3.6.35	960	3.C9.12*	32000	3.167.24	390
3.22.14	960			3.176.17	60
3.13.7*	10000			3.181.11	<10
3.27.2*	12000			3.E5.23	<10
3.34.16*	80				
3.51.17*	12000				
3.129.16*	12000				

† Polyvinyl chloride microtitre plates were coated with rabbit polyclonal anti-rTNF as described for ELISA. Following washing, rTNF, 10^4u/ml, was added, 50µl/well, to all wells and incubated for 1 h at 37°C. Wells were then washed thoroughly with TW20-PBS and anti-rTNF McAbs as supernatant culture fluid or dilutions of ascitic fluid added to the plate, 50µl/well, and incubated for 1 h at 37°C. Wells were again washed thoroughly and then the competing ^{125}I-anti-rTNF McAb added to all wells (50µl containing 1-2x10^5 cpm/well). Following further incubation for 1 h at 37°C and washing, wells were cut from the plate with a hot wire and counted in an LKB gamma counter. Negative control (no competition), 4x10^3 cpm; positive control (full competition, eg ^{125}I-2.33.G2 against cold 2.33.G2) 2x10^2 cpm.
* Indicates that these McAbs partially compete binding of radio-iodinated anti-rTNF McAbs
nu/ml - neutralising units per ml

but nevertheless tend to produce variable results. For instance, purified rTNF (1mg/ml) titred at 5x10^7 units/ml, somewhat higher than the manufacturer's estimate, in our L-929 Cl.10 cells. Immuno-assays, and in particular IRMA, have proved to be valuable alternative assays for many biologically active proteins, eg interferons (15,18). Using a method previously described for HuIFN~ (15), rTNF-specific polyclonal antibodies attached to etched polystyrene beads were first incubated with rTNF (0.1-5000 units/ml; 2-10^{5}pg/ml) and secondly with ^{125}I-anti-rTNF McAb. Typical calibration curves, plotted on a $\log_{10}$-$\log_{10}$ basis, that were generated with ^{125}I-2.33.G2 or ^{125}I-3.101.23 in these IRMA are shown in Fig 1(A). The detection limit of both IRMA was 1 unit (20pg)/ml and calibration curves were linear in the range 1-1000 units/ml. Based on our epitope analysis or rTNF, IRMA were also constructed with different combinations of mutually compatible anti-rTNF McAb pairs. Of many combinations tested, 3.6.10 rTNF McAb in combination with ^{125}I 3.101.23 rTNF McAb (Fig 1(A)) or 2.28.C6 in combination with ^{125}I 3.101.23 (not shown) produced the most sensitive IRMA with calibration curves showing linearity in the range 1-2500 units/ml. An ELISA constructed with rabbit polyclonal anti-rTNF as first

antibody, 2.33.G2 anti-rTNF McAb as second antibody, and biotinylated anti-mouse Ig plus streptavidin biotinylated horseradish peroxidase complex (Amersham International) as amplifying layers on the sandwich assay was also reasonably sensitive for rTNF. A typical calibration curve is shown in Fig 1(B). The detection

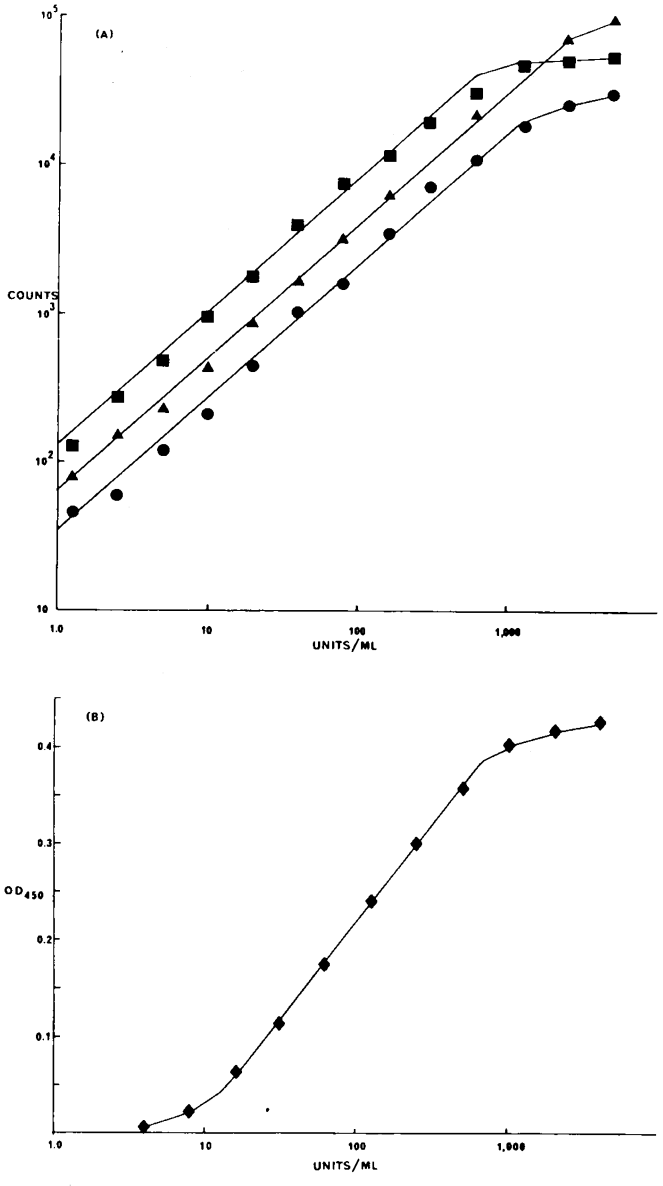

Figure 1(A). Calibration curves generated in IRMA with serial two-fold dilutions of purified rTNF (1-5000 units/ml) using rabbit polyclonal anti-rTNF on the bead and ^{125}I- 3.101.23, ●——●, rabbit polyclonal anti-rTNF on the bead and ^{125}I-2.33.G2, ■——■, and 3.6.10 anti-rTNF McAb on the bead and ^{125}I-3.101.23, ▲——▲. (B) Calibration curve generated in ELISA with serial two-fold dilutions of purified rTNF (1-5000 units/ml) using rabbit polyclonal anti-rTNF on the well and 2.33.G2 anti-rTNF McAb as second antibody, ◆——◆.

limit was approximately 4 units/ml and the calibration curve linear in the range 10-1000 units/ml. Purified 2.33.G2 anti-rTNF McAb could be substituted by other neutralizing anti-rTNF McAbs but non-neutralizing anti-rTNF McAbs were generally less effective (data not shown) for ELISA. Both IRMA and ELISA for rTNF were specific for TNF; neither type of immunoassay detected rLT (Genentech Inc) up to concentrations as high as $5x10^5$ units/ml. The high degree of specificity of these IRMA and ELISA for TNF and the development of equivalent immunoassays for LT (Meager, A., in preparation) will allow separate measurement of TNF and LT where these occur together in biological fluids. This is not now possible using conventional bioassays since the biological properties of TNF and LT appear to be indistinguishable.

A great deal of new information about the structure and functions of TNF has accumulated since the cloning of TNF cDNA (4,5). Newly recognised functions of TNF are still being reported, (eg 19) and more must be learned about the role of TNF in immune regulation and inflammatory reactions. The anti-rTNF McAbs reported in this study should prove useful for definitive studies on the biological activities of TNF as well as for their application for immunoassays and further epitope analysis.

ACKNOWLEDGEMENTS

We are indebted to BASF Knoll AG (Ludwigshafen, FRG) for supplies of highly purified TNF. We thank Dr C. Milstein for the NSO myeloma cell line.

REFERENCES

1. CARSWELL, E.A., OLD, L.J., KASSEL, R.L., GREEN, S., FIORE, N. and WILLIAMSON, B. (1975) An endotoxin-induced serum factor that causes necrosis of tumours. Proc. Natl. Acad. Sci. USA 72, 3666.
2. MÄNNEL, D.N., MOORE, R.N. and MERGENHAGEN, S.E. (1980) Macrophages as a source of tumoricidal activity (tumor-necrotizing factor). Infect. Immun. 30, 523.
3. OLD, L.J. (1985) Tumor necrosis factor (TNF). Science (Washington) 230, 630.
4. PENNICA, D., NEDWIN, G.E., HAYFLICK, J.S., SEEBURG, P.H., DERYNCK, R., PALLADINO, M.A., KOHR, W.J., AGGARWAL, B.B. and GOEDDEL, D.V. (1984) Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin. Nature 312, 724.
5. MARMENOUT, A., FRANSEN, L., TAVERNIER, J., VAN DER HAYDEN, J., TIZARD, R., KAWASHIMA, E., SHAW, A., JOHNSON, M-J., SEMON, D., MÜLLER, R., RUYSSCHAERT, M-R, VAN VLIET, A. and FIERS, W. (1985) Molecular cloning and expression of human tumour necrosis factor and comparison with mouse tumour necrosis factor. Eur. J. Biochem. 152, 515.
6. BROXMEYER, H.E., WILLIAMS, D.E., LU, L., COOPER, S., ANDERSON, S.L., BEYER, G.S., HOFFMAN, R. and RUBIN, B.Y. (1986) The suppressive influences of human tumour necrosis factor on bone marrow hematopoietic progenitor cells from normal donors and patients with leukaemia: synergism of tumour necrosis factor and interferon-γ. J. Immunol. 136, 4487.
7. DEGLIANTONI, G., MURPHY, M., KOBAYASHI, M., FRANCIS, M.K., PERUSSIA, B. and TRINCHIERI, G. (1985) Natural killer (NK) cell-derived hematopoietic colony-inhibiting activity and NK cytotoxic factor. Relationship with tumour necrosis factor and synergism with immune interferon. J. Exp. Med. 162, 1512.
8. ADERKA, D., HOLTMANN, H., TOKER, L. and WALLACH, D. (1986) Tumour necrosis factor induction by Sendai virus. J. Immunol. 136, 2938.

9. SAYERS, T.J., RANSOM, J.H., DENN, A.C., III, HERBERMAN, R.B. and ORTALDO, J.R. (1986) Analysis of a cytostatic lymphokine produced by incubation of lymphocytes with tumour cells: relationship to leukoregulin and distinction from recombinant lymphotoxin, recombinant tumour necrosis factor, and natural killer cytotoxic factor. J. Immunol. 137, 385.
10. MURPHY, M., LOUDON, R., KOBAYASHI, M. and TRINCHIERI, G. (1986) γ-interferon and lymphotoxin, released by activated T cells, synergize to inhibit granulocyte/monocyte colony formation. J. Exp. Med. 164, 263.
11. RUBIN, B.Y., ANDERSON, S.L., SULLIVAN, S.A., WILLIAMSON, B.D., CARSWELL, E.A. and OLD, L.J. (1985) Purification and characterization of a human tumour necrosis factor from the LukII cell line. Proc. Natl. Acad. Sci. USA 82, 6637.
12. HAHN, T., TOKER, L., BUDILOVSKY, S., ADERKA, D., ESHHAR, Z. and WALLACH, D. (1985) Use of monoclonal antibodies to a human cytotoxin for its isolation and for examining the self-induction of resistance to this protein. Proc. Natl. Acad. Sci. USA 82, 3814.
13. KÖHLER, G. and MILSTEIN, C. (1975) Continuous culture of fused cells secreting antibody of predefined specificty. Nature 265, 495.
14. MEAGER, A., PARTI, S., BARWICK, S., SPRAGG, J. and O'HAGAN, K. (1984) Detection of hybridomas secreting monoclonal antibodies to human gamma interferon using a rapid screening technique and specificity of certain monoclonal antibodies to gamma interferon. J. Interferon Res. 4, 619.
15. SECHER, D.S. (1981) Immunoradiometric assay of human leucocyte interferon using monoclonal antibody. Nature 290, 501.
16. GREENWOOD, F.C., HUNTER, W.T. and GLOVER, J.J. (1963) The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J. 89, 114.
17. EY, P.L., PROWSE, S.J. and JENKIN, C.R. (1978) Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using protein A-sepharose. Immunochemistry 15, 429.
18. SCOTT, G.M., ROBINSON, J.A., SECHER, D.S., ASHBURNER, C.M. and ABBOTT, S.R. (1985) Measurement of interferon from *in vitro* stimulated lymphocytes by bioassay and monoclonal antibody-based immunoassay. J. Gen. Virol. 66, 1621.
19. TAKEDA, K., IWAMOTO, S., SUGIMOTO, H., TAKUMA, T., KAWATANI, N., NODA, M., MASAKI, A., MORISE, H., ARIMURA, H. and KONNO, K. (1986) Identity of differentiation inducing factor and tumour necrosis factor. Nature 323, 338.

Address reprint requests:
Dr. A. Meager
Division of Immunology
The National Institute for Biological Standards and Control
Holy Hill
Hamstead
London NW3 6RB
UK

Received for publication November 3, 1986.
Accepted after revisions January 27, 1987.