Myxoma Virus T2 Protein, a Tumor Necrosis Factor (TNF) Receptor Homolog, Is Secreted as a Monomer and Dimer That Each Bind Rabbit TNFα, but the Dimer Is a More Potent TNF Inhibitor*

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The myxoma virus T2 (M-T2) gene expresses a secreted protein that contains significant sequence similarity to the ligand binding domains of the cellular tumor necrosis factor (TNF) receptors, specifically inhibits the cytolytic activity of rabbit TNFα and is an important virulence factor for myxoma virus infection in rabbits. M-T2 protein was overexpressed from vaccinia virus vectors, purified to apparent homogeneity, and found to specifically protect mouse and rabbit cells from lysis by rabbit TNFα at molar ratios comparable with the soluble versions of the host tumor necrosis factor receptors. M-T2 secreted from virus-infected cells is detected as both a monomer and a disulfide-linked dimer, both of which were shown by Scatchard analysis to bind rabbit TNFα (Kd values of 170 pm and 195 pm, respectively), values that are comparable with the affinities of mammalian TNFs with their receptors. In contrast to the rabbit ligand, M-T2 interacts with mouse TNFα with a much lower affinity, Kd of 1.7 nm, and was unable to inhibit the cytolytic activity of this ligand on mouse cells. Although both monomeric and dimeric forms bound rabbit TNFα with comparable affinity, the dimeric M-T2 protein was a far more potent inhibitor of rabbit TNFα, presumably because it can more effectively prevent dimerization of TNF receptors than can the M-T2 monomer.

Poiviruses are a family of double stranded DNA viruses with terminal-inverted repeats and covalently closed hairpin termini that possess genomes large enough to encode for immunomodulatory factors that inhibit or modify antiviral activities such as cytokines that are critical for the host response to infections (1–3). For example, homologs of the cellular receptor for various cytokines are encoded in various poiviral genomes, including receptor homologs of interferon-γ (IFN-γ) (4–7), IFN-α/β (8, 9), interleukin-1 (10, 11), and tumor necrosis factor (TNF) α/β (12, 13) reviewed in Refs. 2, 3, 14, and 15). Myxoma virus, a pathogenic poivirus of European rabbits, encodes two copies of a gene designated T2, because it is the second gene from the viral terminus, which encodes a secreted protein with significant homology to the ligand binding domains of the TNF receptor (TNFR) gene family (12). Infection with myxoma virus results in a severe systemic infection that is invariably fatal for immunocompetent European rabbits (16, 17). However, the majority of rabbits infected with a recombinant myxoma virus in which both copies of the T2 gene were inactivated by insertional mutagenesis were able to mount an effective immune response and recover from infection (12), indicating that T2 is an important virulence factor for myxoma virus. In vitro, the myxoma T2 (M-T2) protein inhibits the cytolytic activities of rabbit TNFα but not mouse or human TNFα on mouse L929-8 cells (18).

TNFα plays a pivotal role in establishing and orchestrating inflammatory and immune responses to infection (19, 20). In addition, TNFα has been shown to have a number of antiviral properties. It inhibits the replication of various DNA and RNA viruses in cultured cells (21) and is directly cytolytic to cells infected with vesicular stomatitis virus (22), herpes simplex virus (23), Newcastle disease virus (24), and various adenovirus deletion mutants (25). In vivo, local expression of TNFα results in a dramatic attenuation of viral pathology following the infection of athymic, nude, or sublethally irradiated mice with recombinant vaccinia viruses expressing TNFα (26), and clearance of the virus is associated with elevated neutrophil numbers within 24 h postinfection (27). Moreover, the importance of TNFα in an antiviral response is reinforced by the fact that adenovirus encodes four proteins that interfere with TNF signal transduction cascades (25, 28).

Biological responses to TNF are initiated when TNFα or TNFβ binds and induces the oligomerization of cellular receptors (29, 30). Two different TNF receptors have been cloned and characterized, designated p55 TNFR and p75 TNFR, each of which binds and responds to both TNFα and TNFβ (31–35). The TNFR superfamily, of which the M-T2 protein is a member, is characterized by the presence of multiple cysteine-rich motifs of about 40 amino acids in their extracellular amino-terminal domain (36, 37). M-T2 protein displays the greatest homology with the p75 TNFR (12), and thus rabbit TNFα and TNFβ would appear to be the most likely candidates for a biological target of M-T2 protein, although other members of the TNF superfamily, including FAS ligand and CD40 ligand cannot be excluded.

The currently favored model is that M-T2 protein acts to inhibit host TNF responses by binding and sequestering either extracellular or cell surface TNF and thereby preventing the activation of cellular TNF receptors. Here we demonstrate that M-T2 protein specifically forms inhibitory complexes with rabbit TNFα and that binding is associated with the ability to inhibit the cytolytic activities of rabbit TNFα on susceptible rabbit and mouse cell lines. M-T2 protein is found to be secreted from virally infected cells as both a monomer and disulfide-linked dimer, both of which bind TNFα with similar affinities. Moreover, the dissociation constant (Kd) values for both

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The abbreviations used are: IFN, interferon; M-T2, myxoma T2; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; tk, thymidine kinase; TNF, tumor necrosis factor; TNFR, TNF receptor.
monomeric and dimeric M-T2 protein, assessed by Scatchard analysis, compare favorably with those of other vertebrate TNFα receptors. However, the dimeric form of M-T2 is far more active than the monomeric form at inhibiting the cytolytic activities of TNFα, most likely because as a diveral inhibitor, the dimer can more effectively inhibit the ability of the TNFα trimeric ligand to induce oligomerization of the cell surface TNF receptors.

**MATERIALS AND METHODS**

Viruses and Cells—VV-601, a derivative of vaccinia virus (strain WR) containing the Escherichia coli lacZ gene inserted into the vaccinia thymidine kinase (tk) locus has been described previously (38). As shown in Fig. 1A, VV-MT2 was engineered to overexpress and secrete the myxoma T2 protein using a synthetic late promoter (18). VMyxlac, a derivative of E. coli, in which the E. coli lacZ gene is inserted between the myxoma growth factor and M9 genes (39), and VMyxT2g, in which both copies of the T2 gene are disrupted (12), are described elsewhere. Viruses were propagated in BGMK cells (provided by S. Dales) cultured in Dulbecco's modified Eagle's medium supplemented with 10% newborn calf serum (Life Technologies, Inc.). Rabbit MT2 cells (provided by L. Guilbert) were cultured in Iscove's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.); mouse L929-8 cells (provided by L. Guilbert) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.); mouse L929-8 cells (obtained from the ATCC) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.); mouse L929-8 cells (obtained from the National Institutes of Health AIDS research and Reference Reagent Program) were grown in RPMI medium supplemented with 10% fetal bovine serum.

**Generation of Rabbit TNFα from a Recombinant Vaccinia Virus—**The plasmid pMJ601T2RaTNF used to make a vaccinia virus that overexpresses secreted TNFα (VV-T2RaTNF) was constructed in which the rabbit TNFα gene was cloned into the vaccinia thymidine kinase (tk) locus has been described previously (38). As shown in Fig. 1A, the plasmid pMJ601T2RaTNF was subcloned into pMA601 to generate pMA601T2RaTNF. The recombinant vaccinia virus VV-T2RaTNF (strain WR) was constructed as described previously (39).

**Purification of Myxoma T2 Protein and Rabbit TNFα**—Myxoma T2 protein was purified from media of VV-MT2-infected BGMK cells in roller bottles in an identical fashion, using pH 7.5Mono-Q anion exchange chromatography followed by pH 6.4 anion exchange chromatography. The M-T2 or rabbit TNFα protein present in the different fractions was confirmed by silver staining and quantitated by A280 measurements.

**Generation of Polyclonal Antibera Against Myxoma T2—**Antiserum was prepared by intradermally injecting rabbits with purified T2 protein (native) in Freund's complete adjuvant or with purified T2 protein resolved on a 12% SDS-polyacrylamide gel (denatured) with acrylamide as adjuvant using standard procedures (42). Immunoblots confirmed that the polyclonal antiserum recognized T2 protein from supernatants of myxoma- and VV-MT2-infected cells and did not cross-react with any proteins present in supernatants from cells infected with the T2-minus myxoma virus (VMyxT2g) or control VV-601.

**Purification of Myxoma T2 Protein Secreted from Virally Infected Cells—** Western analysis was used to estimate the amount of M-T2 protein secreted from from VV-Myxlac and VV-MT2-infected cells, probing with a 1:5000 dilution of rabbit anti-M-T2 antisera followed by a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Bio-Rad). The intensities of the bands visualized were measured by scanning using a Joyce Loebel Chromoscan 3 densitometer. The results of four separate Western blots were averaged to determine the amount of M-T2 protein present in supernatants, using various titrations of purified M-T2 protein as the standard.

**Co-immunoprecipitation of M-T2 and Rabbit TNFα—**To detect M-T2 and rabbit TNFα proteins secreted from recombinant vaccinia virus-infected cells, confluent BGMK cell monolayers in six-well dishes were infected with virus for 1 h, followed by a multiplicity of 10 plaque-forming units per cell. Next, Dulbecco's modified Eagle's medium, 10% newborn calf serum was added, and the infection was allowed to proceed at 37°C for 4 h. Cells were washed with PBS and incubated 16 h in 0.5 ml of serum-free, Met-free, Cys-free Dulbecco's modified Eagle's medium containing 150 μCi of [35S]Met/Cys protein label ([35S]Met/Cys mixture, DuPont NEN), after which supernatants were collected. 100-μl supernatants from VV-MT2, VV-T2RaTNF, or VV-601-infected cells were incubated for 1 h at 4°C, after which 1 μg of purified goat anti-rabbit TNFα antibody (PharMingen) or a 1:200 dilution of rabbit anti-T2 antisera was added and the mixture was incubated for 1 h further at 4°C. The beads were collected by centrifugation at 10,000 g for 1 min at 4°C, and the immunocomplexes were washed three times with lysis buffer, resuspended in 50 μl of Laemmli sample buffer, and resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was dried onto Whatman 3MM chromatography paper and analyzed by autoradiography. Alternatively, 25 ng of purified T2 protein was incubated with 100 μl of [35S]Met/Cys-labeled supernatant from VV-T2RaTNF-infected cells, and co-immunoprecipitations were performed as above using a 1:200 dilution of rabbit anti-M-T2 antisera.

**GeF Filtration Chromatography of Purified Myxoma T2 Protein—**A Superdex 200 gel filtration column (Pharmacia) was calibrated using the markers ferritin, catalase, aldolase, and bovine serum albumin (Pharmacia) as size standards. Purified T2 protein was loaded onto the calibrated Sephadex 200 gel filtration column equilibrated with PBS, and fractions were collected. Fractions containing protein were resolved by SDS-PAGE under reducing (50 μM Tris-Cl, pH 6.8, 10 mM dithiothreitol, 2% SDS, 0.1% bromphenol blue, 10% glycerol sample loading buffer) or non-reducing (50 μM Tris-Cl, pH 6.8, 2% SDS, 0.1% bromphenol blue, 10% glycerol sample loading buffer), transferred to nitrocellulose, and probed with a 1:5000 dilution of rabbit anti-M-T2 antisera as described (38).

**Sedimentation Equilibrium Analysis—**Sedimentation equilibrium studies were performed on a Beckman Spinco model E analytical ultracentrifuge using absorbance optics. The run was performed at 8000 rpm in PBS at 20°C. The molecular weight (M) was calculated using the equation:

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M = \frac{2RT}{(1 - \phi) \rho \bar{d} \bar{v}} \cdot \frac{dC}{dR^2}
\]

where \(R\) is the universal gas constant, \(T\) is the temperature in Kelvin, \(\phi\) is the partial specific volume, \(\rho\) is the solvent density, \(\bar{d}\) is the angular velocity, and \(\bar{v}\) is the slope from the plot in Fig. 4. Based on the amino acid composition of M-T2 protein, \(\bar{d}\) was calculated to be 0.69. As M-T2 is glycosylated, \(\bar{d}\) for the glycoprotein was calculated to be 0.66 using the formula \(L_1 + M_1 + M_2\), where \(L_1\), \(M_1\), and \(M_2\) are the masses and partial specific volume of the protein and the sugar component, respectively.

**Solid Phase M-T2 Binding Assay—**Purified rabbit TNFα, human TNFα (Quality Controlled Bioc hemicals), and mouse TNFα (PharMin-
Purification of Myxoma T2 Protein and Rabbit TNFα—Recombinant vaccinia viruses were engineered to express myxoma T2 protein (VV-MT2) and rabbit TNFα (VV-RaTNF) genes (45) (Fig. 1A). Previously, the complete sequence for the rabbit TNFα cDNA, including the 79-amino acid leader, was placed under the control of the synthetic late promoter in the recombinant virus VV-RaTNF (18). However, TNFα was not efficiently expressed as a secreted ligand from cells infected with VV-RaTNF, so instead the recombinant vaccinia virus VV-T2RaTNF was constructed in which the precursor N-terminal to the mature rabbit TNFα was excised and replaced with the 16-amino acid leader sequence of myxoma T2 protein. Pro-myxoma T2 protein was found by N-terminal sequencing to be cleaved at its predicted signal cleavage site (Fig. 1B) and purity of rabbit TNFα was checked by SDS-PAGE (Fig. 1C).

RESULTS
Purification of Myxoma T2 Protein and Rabbit TNFα—Recombinant vaccinia viruses were engineered to express myxoma T2 protein (VV-MT2) and rabbit TNFα (VV-RaTNF) and VV-T2RaTNF, in which M-T2 sequence, the entire rabbit TNFα coding sequence, or the M2 leader sequence/mature rabbit TNFα coding sequence was placed under the control of the vaccinia synthetic late promoter (SLP) and inserted together with E. coli lacZ into the tk locus of vaccinia-WR. Asterisks indicate predicted N-linked glycosylation sites, oval boxes designate the four cysteine-rich repeats of M-T2, arrowheads designate predicted N-terminal cleavage sites, and TIR designates the terminal inverted repeats. B, purification of M-T2 displaying proteins present in crude supernatants from VV-601 (lane 1) or VV-MT2-infected cells (lane 2), purity of M2 after the first anion exchange chromatography, pH 7.5 (lane 3), followed by a second anion exchange chromatography, pH 6.4 (lane 4), and corresponding fraction from control VV-601 supernatants (lane 5). C, purification of secreted rabbit TNFα from supernatants from VV-T2RaTNF-infected cells (lane 1) and purity of rabbit TNFα after the first anion exchange chromatography, pH 7.5 (lane 2), followed by a second anion exchange chromatography, pH 6.4 (lane 3). Size standards of 66.2, 45, 31, 21.5, and 14.4 kDa are marked on panels B and C.

Fig. 1. Structure of recombinant vaccinia viruses expressing M-T2 and rabbit TNFα and purification of M2 and rabbit TNFα proteins. A, structure of VV-601, VV-MT2, VV-RaTNF, and VV-T2RaTNF in which M-T2 sequence, the entire rabbit TNFα coding sequence, or the M2 leader sequence/mature rabbit TNFα coding sequence was placed under the control of the vaccinia synthetic late promoter (SLP) and inserted together with E. coli lacZ into the tk locus of vaccinia-WR. Asterisks indicate predicted N-linked glycosylation sites, oval boxes designate the four cysteine-rich repeats of M-T2, arrowheads designate predicted N-terminal cleavage sites, and TIR designates the terminal inverted repeats. B, purification of M-T2 displaying proteins present in crude supernatants from VV-601 (lane 1) or VV-MT2-infected cells (lane 2), purity of M2 after the first anion exchange chromatography, pH 7.5 (lane 3), followed by a second anion exchange chromatography, pH 6.4 (lane 4), and corresponding fraction from control VV-601 supernatants (lane 5). C, purification of secreted rabbit TNFα from supernatants from VV-T2RaTNF-infected cells (lane 1) and purity of rabbit TNFα after the first anion exchange chromatography, pH 7.5 (lane 2), followed by a second anion exchange chromatography, pH 6.4 (lane 3). Size standards of 66.2, 45, 31, 21.5, and 14.4 kDa are marked on panels B and C.

M-T2 Inhibition of Binding of 125I-Labeled Rabbit TNFα to RK-13 and RL-5 Cells—To measure specific binding of 125I-labeled rabbit TNFα to RL-5 (rabbit T-cell lymphoma) cells, 2 x 106 cells were seeded in microcentrifuge tubes in 100 μl of RPMI medium supplemented with 10% fetal bovine serum, 500 pM 125I-labeled rabbit TNFα in the presence or absence of a 100-fold excess of cold TNFα, for 1 h at 4°C. The cell suspension was then layered over 250 μl of transport oil (15% silicone 550 oil, specific gravity 1.032 g/ml) in microcentrifuge tubes; the cells were pelleted through the oil by centrifugation at 10,000 g for 1 min; and the oil and cell pellet were washed three times with water. The cell pellet was resuspended in 0.5% SDS, and the radioactivity present in the cell pellet was measured on a Beckman 5500 γ counter. All assays were performed in triplicate.

To measure TNF-binding to RK-13 (rabbit fibroblast) cells, 1 x 106 RK-13 cells seeded in six-well plates were incubated in 200 μl of Dulbecco’s modified Eagle’s medium supplemented with 10% newborn calf serum. 125I-labeled rabbit TNFα was added at a concentration of 500 pM in the presence or absence of a 100-fold excess of cold TNFα to measure specific binding, or in the presence of 20 nM purified M-T2 protein, and cells were incubated 1 h at 4°C with gentle agitation. Cells were then washed three times with PBS and resuspended in 200 μl of 0.5% SDS, and the radioactivity remaining in the sample was measured on a Beckman γ counter. All assays were performed in triplicate.

M-T2 Inhibition of TNFα-Induced Cytolysis—The ability of purified myxoma T2 protein to protect rabbit or mouse cells from TNFα-induced cytotoxicity was measured as described previously (18) except that for RK-13 cells viability was measured by crystal violet staining as described (44). TNFα (rabbit, mouse, human) was used at concentrations for which the cell death end point value was greater than 95% for L929-B cells and greater than 65% for RK-13 cells. Results are expressed as percentage of viable cells compared with untreated control cells, the mean of quadruplicate samples calculated by the following formula: (OD experimental – OD maximum killing)/(OD maximum viability – OD maximum killing) x 100, where OD represents optical density. When protection was expressed as an increase in percentage of viability (% viability), the formula (% viability experimental – % viability in the absence of M-T2) was used. In calculating M-T2:TNFα ratios, molarities were calculated for the M-T2 monomer and TNFα trimer unless stated otherwise.

TFN Inhibition by Myxoma Virus Dimeric TNF Receptor (M-T2)
rabbit TNFα. Fractons were pooled and reapplied to the Mono-Q column at pH 6.4, and then immunoprecipitated with anti-rabbit TNFα antibody (lanes 1–3 and lanes 7–9) or polyclonal rabbit anti-MT2 antisera (lanes 4–6 and lanes 10–12) as described under "Materials and Methods" and analyzed by SDS-PAGE and autoradiography. Size standards of 112, 84, 53.2, 34.9, 28.7, and 20.5 kDa are indicated.

(lane 3). When reapplied to the anion exchange column at pH 6.4, a single protein band was observed on silver-stained SDS-PAGE gels in fractions collected at 100–150 mM NaCl, with no protein bands seen from supernatants from vector control virus, VV601, processed in identical fashion (Fig. 1B, lanes 4 and 5, respectively).

The purification of rabbit TNFα is illustrated in Fig. 1C, starting from supernatants of VV-T2RaTNF-infected monolayers (lane 1). At pH 7.5, rabbit TNFα eluted from the Mono-Q column at 300–340 mM NaCl (Fig. 1C, lane 2). TNF-containing fractions were pooled and reapplied to the Mono-Q column at pH 6.4, and rabbit TNFα eluted at 260–275 mM NaCl as a single 18-kDa species when visualized on silver-stained gels (Fig. 1C, lane 3).

The amount of M-T2 protein secreted into supernatants from BGMK cells infected with either VV-MT2 or VV-myxlac virus was quantitated from densitometric scans of Western blots, using various titrations of purified M-T2 protein as standards. As expected, the amount of M-T2 protein secreted from VV-MT2-infected cells was significantly higher than from myxoma-infected cells (1.0 ± 0.4) × 10⁶ molecules/24 h. M-T2 secreted per VV-MT2-infected cell compared with (2.8 ± 0.5) × 10⁶ molecules/24 h of M-T2 secreted per VV-myxlac infected cell. In VV-MT2, the M-T2 gene is under the control of a very strong synthetic late promoter (40), which accounts for the enhanced expression compared with the standard (early) promoter in the parental myxoma virus.

Myxoma T2 Protein Specifically Complexes with Rabbit TNFα—To determine whether M-T2 protein and rabbit TNFα physically associate, immunoprecipitation experiments were performed. M-T2 polyclonal antibody correctly immunoprecipitates native M-T2 protein, with an apparent molecular mass of 60 kDa, from supernatants from 35S-Met/Cys-labeled VV-MT2 infected cells (Fig. 2, lanes 4 and 10) but not from control VV-601- or VV-T2RaTNF-infected cells (Fig. 2, lanes 5, 11, and 12). Similarly, anti-rabbit TNFα antibody only immunoprecipitates rabbit TNFα, an 18-kDa protein from supernatants from VV-T2RaTNF-infected cells (lanes 2 and 8) but not from VV-601- or VV-MT2-infected cells (lanes 1, 7, and 9). When supernatants from VV-601- or VV-MT2-infected cells were co-precipitated with supernatants from VV-T2RaTNF-infected cells for 1 h and then immunoprecipitated with anti-rabbit TNFα antibody, radiolabeled proteins of sizes identical to myxoma T2 protein and rabbit TNFα were co-precipitated in VV-MT2/VV-T2RaTNF mixtures (Fig. 2, lane 3). Similarly, anti-myxoma T2 antisera was able to co-precipitate both rabbit TNFα and myxoma T2 proteins from mixtures of VV-MT2/VV-T2RaTNF (lane 6). Thus, both polyclonal antisera against myxoma T2 protein and anti-rabbit TNFα antibody were each able to immunoprecipitate the complex between rabbit TNFα and myxoma T2 protein. In contrast, anti-myxoma T2 antisera was unable to co-immunoprecipitate 35S-radiolabeled myxoma T2 and 125I-radiolabeled mouse TNFα (data not shown).

Native Size of M-T2 Protein Secreted from Virus-infected Cells—Purified T2 protein was fractionated by Superdex 200 gel filtration chromatography to determine whether it is expressed as a monomer or oligomer. N-terminal sequencing had shown this to be a pure preparation of M-T2. However, as shown in Fig. 3A, M-T2 protein eluted from the column as two distinct peaks, estimated to be 140–156 kDa and 56–65 kDa in size, based on size standards of a variety of monomeric proteins. When samples from these two peaks were analyzed by SDS-PAGE under reducing conditions and probed by Western blotting, anti-M-T2 antisera recognized only a single 55–60-kDa protein band in fractions from both peaks (Fig. 3A, inset), indicating that the first peak to elute from the Superdex column is most likely an oligomeric form of the second peak. When analyzed under nonreducing conditions, the first eluted M-T2 peak electrophoresed as a single 87–90-kDa band on SDS-polyacrylamide gels (Fig. 3B, lane 1), whereas the second M-T2 peak eluted from the Superdex column electrophoresed as a single 55–59-kDa species (lane 2). However, both M-T2 species co-migrated at approximately 60 kDa when the samples were reduced with dithiothreitol prior to electrophoresis, suggesting that the two M-T2 peaks detected by Superdex chromatography (Fig. 3A) are the monomeric and disulfide-linked dimeric forms of the same M-T2 protein. To confirm this result and to resolve the discrepancies in the apparent molecular mass of M-T2 protein determined by SDS-PAGE and gel filtration chromatography, the oligomeric protein from the first Superdex 200 M-T2 peak was examined by mass spectrometry and ultracentrifugation studies. When analyzed by mass spectrometry, a mass of 80.9 kDa was calculated for the larger species, confirming that it is in fact a dimer of a 40.5-kDa species. Moreover, by ultracentrifugation sedimentation analysis, the plot of ln C versus r² yielded a straight line, indicating that this larger
M-T2 peak from the Superdex 200 column is a single species with a calculated molecular mass of approximately 79 kDa (Fig. 4). Scatchard Analysis of M-T2 Binding to TNFα—To quantitate the affinity of M-T2 protein for rabbit TNFα, solid phase binding assays were performed, followed by Scatchard analysis. Experiments with M-T2 protein containing both monomeric and dimeric forms yielded a single slope (not shown), indicative of a single affinity binding site, but to substantiate this interpretation, binding analysis was carried out on the purified M-T2 monomer and dimer individually. The binding of 125I-radiolabeled rabbit TNFα with both dimeric and monomeric M-T2 protein, separated by gel filtration chromatography (Fig. 3A), was therefore examined using various concentrations of labeled TNFα either in the presence of absence of excess unlabeled ligand. The specific binding of rabbit TNFα increased with increasing concentration of labeled ligand and reached saturation at 500 pm for both dimeric and monomeric M-T2 (Fig. 5A and B). For both forms of M-T2 the Scatchard plot of the binding data was consistent with a single class of high affinity binding sites with similar affinities, a calculated Kd of 195 pm with a correlation coefficient of 0.95 for dimeric M-T2 (Fig. 5A), and a Kd of 170 pm with a correlation coefficient of 0.96 for monomeric M-T2 (Fig. 5B). Saturable binding was also observed between M-T2 protein, containing both monomeric and dimeric species, and 125I-radiolabeled mouse TNFα but only at a higher concentration in the nM range (Fig. 5C), while no binding at all was observed with radiolabeled human TNFα (Fig. 5D). The affinity of myxoma T2 and mouse TNFα was an order of magnitude lower than the affinity of M-T2 with rabbit TNFα, and the Kd was calculated to be 1.7 nM with a correlation coefficient on the Scatchard plot of 0.98. Thus, the biological specificity of M-T2 for rabbit TNFα (18) is consistent with this physical binding data.

Rabbit TNF receptors have not yet been cloned or characterized. Attempts to determine the Kd of cognate rabbit TNF receptors with rabbit TNFα were unsuccessful because of the low number of TNF receptors present on all rabbit cell lines screened. Instead, we tested to see whether M-T2 protein can inhibit the binding of 125I-labeled rabbit TNFα to cellular receptors on two rabbit cell lines that exhibited some TNF binding. Rabbit RL-5 cells, a CD4+ T cell lymphoma cell line, and RK-13 cells, a fibroblast kidney line, were incubated with 500 pm 125I-rabbit TNFα in the presence or absence of a 100-fold excess of cold TNF to determine specific binding. As shown in Fig. 6, a 400-fold molar ratio excess of purified M-T2 protein, 200 nm, was able to effectively block specific binding of rabbit TNFα to both RL-5 cells (lane 2) and RK-13 cells (lane 6). Similarly, even crude supernatant from VV-MT2-infected BGMK cells, containing approximately 50 nm M-T2 protein, was also able to inhibit binding of radiolabeled rabbit TNFα to RL-5 cells (lane 3), whereas control supernatant from VV-601-infected cells (lane 4) had no effect. Thus, we conclude that M-T2 protein as secreted from infected cells actually blocks the binding of rabbit TNFα to the cell surface of rabbit fibroblasts and lymphocytes.

Myxoma T2 Specifically Inhibits Rabbit but Not Mouse TNFα—Previously we had shown that myxoma T2 protein present in crude supernatants from VV-MT2-infected cells was able to inhibit the cytolytic activities of rabbit TNFα on mouse L929-8 cells but not the mouse or human ligands (18). Since myxoma T2 was found by Scatchard analysis to bind both rabbit and mouse TNFα, albeit with different affinities (Fig. 5), anion exchange chromatography-purified myxoma T2 protein, containing both monomers and dimers, was tested for its abil-
TNF Inhibition by Myxoma Virus Dimeric TNF Receptor (M-T2)

Fig. 6. M-T2 inhibition of the binding of rabbit TNFα to rabbit membrane TNFRs. 2 × 10⁶ RL-5 cells (speckled box) or 1 × 10⁶ RK-13 cells (open box) were incubated with 500 pm ¹²⁵I-labeled rabbit TNFα in the presence and absence of a 100-fold excess of cold TNFα to determine ligand-specific binding to the cell surface. 200 nm purified M-T2 protein, or 10% (v/v) supernatant from VV-601-infected or VV-MT2-infected cells (containing approximately 50 nM MT2 protein) was added as indicated, as outlined under “Materials and Methods.” Standard deviations of triplicate assays are shown.

Fig. 7. M-T2 protects against cytolysis by rabbit but not mouse TNFα. Actinomycin D-sensitized L929-8 cells (A and B) or RK-13 cells (C) were incubated at varying molar ratios of M-T2 protein and mouse TNFα (A) or rabbit TNFα (B and C) as described under “Materials and Methods” and the percentage increase in cell viability (A) or percentage of viability of cells (B and C) determined by neutral red uptake (A and B) or crystal violet staining (C). A monomeric size of 40 kDa was calculated for M-T2, while the trimeric size of 54 kDa for rabbit TNFα and 51 kDa for mouse TNFα was used. Points represent the mean of quadruplicate determinations.

Fig. 8. A) M-T2 protein is a monomer at 80 kDa and is therefore a much more effective inhibitor of TNFα than is monomeric M-T2. B) M-T2 protein is a monomer at 80 kDa and is therefore a much more effective inhibitor of TNFα than is monomeric M-T2. C) M-T2 protein is a monomer at 80 kDa and is therefore a much more effective inhibitor of TNFα than is monomeric M-T2.

DISCUSSION

Myxoma virus, a member of the poxvirus family of viruses, encodes a wide spectrum of immunomodulatory proteins, including several secreted homologs of cytokine receptors (46, 48, 50, 51). Myxoma virus, a member of the poxvirus family of viruses, encodes a wide spectrum of immunomodulatory proteins, including several secreted homologs of cytokine receptors (46, 48, 50, 51).
Tolytic activity of rabbit TNF

Previously we have shown that M-T2 protein inhibits the cytokine from participating in the local antiviral response (12). M-T2 protein, which is encoded by two copies of the T2 gene, possesses homology to the extracellular ligand binding domains of the TNFRs and presumably acts as a TNF antagonist of the p55 and p75 TNFRs with their cognate TNF-α and TNF-β.

Figure 8. Protection against TNF-α induced cytolysis by monomeric and dimeric M-T2 protein. A, actinomycin D-sensitized L929-8 cells were incubated with increasing amounts of rabbit TNF-α alone (circles) or in the presence of 5 nM dimeric M-T2 (squares) or 10 nM monomeric M-T2 (triangles), and cell viability was determined by neutral red uptake as outlined under “Materials and Methods.” Each point represents the mean of quadruplicate determinations. B, actinomycin D-sensitized L929-8 cells were incubated with increasing amounts of rabbit TNF-α alone (circles) or in the presence of 200 nM monomeric M-T2 (triangles). C, 35S-labeled supernatants from VV-T2RaTNF-infected cells were incubated with no MT2 (lanes 1 and 2) or 25 ng of dimeric M-T2 (lane 3) or monomeric M-T2 (lane 4), immunoprecipitated with rabbit anti-MT2 antisera (lanes 2–4) or anti-rabbit TNF-α antibody (lane 1), and analyzed by SDS-PAGE as outlined under “Materials and Methods.” The autoradiogram showing immunoprecipitated rabbit TNF-α is displayed.

M-T2 protein, which is encoded by two copies of the T2 gene, possesses homology to the extracellular ligand binding domains of the TNFRs and presumably acts as a TNF antagonist, binding and sequestering host TNF, thus preventing this ligand from participating in the local antiviral response (12). Previously we have shown that M-T2 protein inhibits the cytolitic activity of rabbit TNF-α on mouse L929-8 cells, but not mouse or human TNF-α (18). Here we show that M-T2 and rabbit TNF-α form stable inhibitory complexes and that the dimeric form of M-T2 is the superior inhibitor of TNF-α biological activity.

Biochemical characterization of M-T2 protein revealed that it is secreted from virus-infected cells as both a 40-kDa monomer and an 80-kDa disulfide-linked dimer. Because of discrepancies in the apparent molecular weights between SDS-PAGE and gel filtration analysis, we used mass spectrometry and ultracentrifugation sedimentation equilibrium techniques to confirm the size and oligomeric status of M-T2. M-T2 protein was detected as both a monomer, approximately 40 kDa in size, and an 80.9-kDa dimer, as determined by mass spectrometry. Moreover, sedimentation equilibrium analysis of the dimer indicated that it was a single stable species, of approximately 79 kDa. The calculated molecular mass from amino acid composition of unmodified, monomeric, secreted M-T2 is predicted to be 33 kDa. The difference between the theoretical and observed molecular masses can be accounted for by N-linked glycosylation at one or more of the four predicted glycosylation sites on the M-T2 protein.2

Both the monomeric and dimeric forms of M-T2 protein form stable complexes with rabbit TNF-α with similar binding affinities. High affinity binding with a Kd value of 170 and 195 pm were observed between rabbit TNF-α and monomeric and dimeric M-T2, respectively, by solid phase binding and Scatchard analysis. Because of the low number of TNFRs present on rabbit cell lines tested, we were unable to accurately quantitate the affinity of rabbit TNF-α for rabbit cell surface TNFRs, but reported Kd values for the binding of human and mouse TNF-α to the p75 TNFR range from 50 to 500 pm (32, 48–50), whereas the p55 TNFR displays a somewhat lower affinity with reported Kd values ranging from 130 to 2300 pm (50–52). The calculated Kd for myxoma T2 protein with rabbit TNF-α thus compares favorably with the Kd values determined for the human and mouse TNFRs with their cognate TNF-αs.

Soluble TNFRs, consisting of truncated versions of the extracellular ligand binding domains of the p55 and the p75 receptors, bind TNF-α with an affinity comparable with the full-length membrane receptors (48, 51, 53). This is in contrast to many soluble receptors, including the IFN-γ receptor, which often bind their respective ligands with lower affinity than the membrane-bound counterpart (54, 55). The myxoma virus homolog of the IFN-γ receptor (M-T7), proposed to act in an analogous fashion to M-T2, binds rabbit IFN-γ with a Kd of 1.2 nm (5), which is 6–7-fold lower affinity than M-T2 for rabbit TNF-α. However, M-T7 is by far the most abundantly secreted viral protein from myxoma-infected cells (4), with 4 × 107 molecules secreted per infected cell in the first few hours of myxoma infection (5), whereas only 3 × 106 molecules of M-T2 are secreted per infected cell once the course of a single infection cycle. Moreover a wide variety in the number of TNF receptors present on various cell types has been reported, ranging from 100 to 10,000 copies/cell (32, 56, 57), which may be accounted for by a low, constitutive expression of p55 TNFR (1000 copies/cell (57)) and an inducible level of p75 TNFR expression (32, 57). It is the p55 TNFR which is believed to signal the vast majority of the pathogen response activities of TNF (33, 34) including cytotoxicity (35, 58) and antiviral activity (59). Thus, we presume that M-T2 must block ligand interaction with the presumptive rabbit version of the p55 TNFR to inhibit the direct cytolitic or antiviral activities of TNF-α. The level of M-T2 protein secreted from infected cells is still in vast excess over the estimated number of p55 TNFRs present on TNF-α-responsive cells, and levels of M-T2 secreted by myxoma virus should therefore be sufficient to inhibit TNF-α within the microenvironment of virus-infected tissues.

Full-length p55 receptor has an equivalent affinity for both TNF-α and TNF-β, while the soluble version of the same receptor displays a 50–60-fold lower affinity for TNF-β (53, 60). A comparison of M-T2 affinity for rabbit TNF-α and TNF-β would indicate whether TNF-β is also a candidate for M-T2 inhibitory action as well. However, although the rabbit TNF gene locus has been sequenced, and the amino acid sequence of rabbit

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TNFβ has been deduced from the genomic sequence (61), all our attempts at isolating rabbit TNFβ cDNA using this sequence were unsuccessful; thus, we cannot at this point comment on whether rabbit TNFβ is a biologically relevant target for M-T2 in addition to rabbit TNFα.

Although solid phase saturable binding between mouse TNFα and myxoma T2 protein was measured (Kd of 1.7 nm), we were unable to co-immunoprecipitate M-T2 protein complexed with mouse TNFα (data not shown). Moreover, purified myxoma T2 protein was unable to protect mouse L929-8 cells from lysis by mouse TNF even at M-T2:TNFα molar ratios of 50,000:1, confirming our earlier observations on the species specificity of M-T2 (18) and further reinforcing the idea that binding and inhibition are not synonymous for this class of viral cytokine inhibitors.

On the other hand, purified M-T2 protein was able to effectively protect mouse and rabbit cells from lysis by rabbit TNFα, with 50% protection observed at 50-fold molar excess of M-T2 protein and complete protection observed at 300-fold molar excess of M-T2. M-T2 protein was even more effective at protecting rabbit RK-13 cells from lysis by rabbit TNFα in that a 50% inhibition of TNF cytotoxicity was observed at only a 10-fold molar excess of M-T2, and complete protection was seen at 50-fold excess. Thus, M-T2 protein is able to prevent rabbit TNFα signaling not only through heterologous mouse TNFRs but also to the homologous rabbit TNFRs. One might predict that rabbit TNF receptors would have a higher affinity for rabbit TNFα than do the mouse receptors and that M-T2 protein would be better at competing with mouse TNFRs than rabbit receptors for binding rabbit TNFα. However, it should be cautioned that L929-8 cells were selected for their hypersensitivity to TNF (including the mouse, human, and rabbit ligand), and the increased amounts of M-T2 protein required for protection may simply reflect a heightened capacity of L929-8 cells to effect a TNF signaling cascade with relatively fewer molecules of TNF binding to surface receptors.

A 10–500-fold molar excess of human p55 soluble TNFR is required to inhibit the cytolytic activity of human TNFα on various cell lines (51, 53, 62, 63), which compares favorably with the ratios of M-T2 required to inhibit rabbit TNFα. In comparing the ability of the human soluble p55 and p75 receptors to inhibit the cytolytic effects of human TNFα on L929-8 cells, Hale et al. (62) found that the soluble p55 receptor was a 10-fold more potent inhibitor than the soluble p75 receptor. Moreover, the soluble p55 receptor was 100-fold more effective than soluble p75 receptor at blocking binding of TNFα to U-937 cells (64). The kinetics of association and dissociation of TNF with the p75 TNFR is much more rapid than with the p55 TNFR (65), which may account for its reduced effectiveness as a TNFα inhibitor. Although M-T2 and the related S-T2 protein from Shope fibroma virus show greater sequence similarity with the p75 TNFR than the p55 TNFR (66), we show here that M-T2 protein is as potent an inhibitor of TNFα cytosis as is the soluble p55 TNFR.

According to our model of M-T2 action, M-T2 sequesters TNFα into stable inhibitory complexes and prevents its association with receptors present on the cell surface. However, another possibility is that M-T2 binding of TNFα may not necessarily occlude binding of the TNFα trimer to cellular receptors but may prevent receptor-bound TNFα from signaling, perhaps by preventing the subsequent clustering of membrane TNF receptors. However, we have shown that a 400-fold molar excess of M-T2 was able to effectively block binding of 125I-labeled rabbit TNFα to both rabbit lymphocytes and fibroblasts to near background levels. At this molar ratio of M-T2: TNFα, RK-13 fibroblast cells show complete protection against lysis by TNFα, although the same experiment could not be performed with RL5 lymphocytes because they are not sensitive to TNF cytosis. As the protective capabilities of M-T2 correlate with its ability to inhibit binding of TNFα to membrane receptors, we conclude that M-T2 inhibits the cytolytic activities of TNFα by physically preventing TNFα from associating with its cognate TNF membrane receptors.

Although both the monomeric and dimeric species of M-T2 bind rabbit TNFα with similar affinities, the dimeric form of M-T2 was much more effective at inhibiting cytosis of L929-8 cells by rabbit TNFα than the monomeric form. A 25-fold molar excess of dimeric M-T2 protein, compared with a 2000-fold excess of monomeric M-T2 protein, was required to completely inhibit the cytolytic activity of rabbit TNFα on L929-8 cells. Similar observations have been made with monomeric and dimeric forms of the cellular soluble TNFRs. Dimeric soluble TNFRs, consisting of chimeric immunofusion proteins of the extracellular ligand binding domains with human IgG Fc, are much more active at inhibiting the biological activities of TNFα than the corresponding soluble receptors (51, 64, 63, 67–69). Concentrations of dimeric soluble p55 TNFR equimolar to TNFα are sufficient to neutralize the cytolytic activity of TNFα on WEHI-164, SK MEL 109, and KYM-1D4 cells, whereas a 10–1000-fold excess of soluble p55 TNFR monomer was required to achieve similar levels of inhibition (51, 70, 71). Part of the enhanced inhibitory activity of the bivalent immunofusion proteins can be accounted for by their higher affinity for TNFα than the monovalent soluble TNFRs (51, 54, 68), which most likely reflects an increased avidity of the bivalent construct for its ligand. However, the difference between the dimeric constructs and soluble receptors in inhibiting TNFα activity (10–1000-fold) is significantly greater than their differences in binding affinity for TNFα (6–8 fold). Clustering of membrane-bound TNFRs, either by dimerization or trimerization, is believed to be important in establishing a downstream signal (29, 30, 32). Dimerization of erythropoietin/p55 or p75 TNFR chimeras is sufficient to elicit a biological response (72, 73), although receptor dimerization may be less effective at initiating signal transduction cascades than trimerization (32, 73). Dimeric soluble TNFRs could simultaneously block two receptor-binding sites on a single TNFα trimer, thus rendering TNFα unable to even dimerize cell surface TNF receptors (67), whereas two soluble TNFRs would have to bind a TNFα trimer in order to similarly inactivate it from dimerizing receptors. Dimeric M-T2 protein thus could act in an analogous fashion, by preventing even dimerization of cellular TNFRs, which would account for its 100-fold greater inhibitory activity over that of the monomeric M-T2 protein. Thus, we conclude that poxviruses exploit the greater inhibitory capacity of linked receptor dimers, thus strengthening the concept that the effective blockade of cytokine function can be achieved by sequestering important ligands such as TNF into inhibitory complexes that disallow receptor oligomerization and subsequent intracellular signaling cascades.

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