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Adenovirus E3 14.7K Protein Functions in the Absence of Other Adenovirus Proteins To Protect Transfected Cells from Tumor Necrosis Factor Cytolysis

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A 14,700-kDa protein (14.7K) encoded by the E3 region of adenovirus has been shown to protect adenovirus-infected mouse C3HA cells from lysis by tumor necrosis factor (TNF) (L. R. Gooding, L. W. Elmore, A. E. Tollefson, H. A. Brady, and W. S. M. Wold, Cell 53:341–346, 1988). These infected cells are sensitized to TNF by expression of the adenovirus E1A proteins (P. Duerksen-Hughes, W. S. M. Wold, and L. R. Gooding, J. Immunol. 143:4193–4200, 1989). In this study we show that 14.7K suppresses TNF cytolysis independently of adenovirus infection. Mouse C3HA and C127 cells were transfected with the 14.7K gene controlled by the mouse metallothionein promoter, and permanent 14.7K-expressing cell lines were tested for sensitivity to TNF cytolysis. Transfected cells which were sensitized to TNF either by inhibitors of protein synthesis, microfilament-destabilizing agents, or adenovirus infection were found to be resistant to TNF cytolysis. Two monoclonal antibodies were isolated and used to quantitate 14.7K in transfected and infected cells. Enzyme-linked immunosorbent assay (ELISA) analysis with these monoclonal antibodies and 14.7K immunoblots showed that 14.7K expression can be induced with cadmium in C3HA and C127 transfectants. The 14.7K induction correlated with a dose-dependent decrease in sensitivity to TNF cytotoxicity. The 14.7K protein does not substantially alter cell surface TNF receptor numbers or affinity on C3HA mouse fibroblasts, as determined by Scatchard analysis of ^{125}I-TNF binding. The 14.7K protein also does not alter TNF signal transduction in general, because TNF induction of cell surface class I major histocompatibility complex molecules on 14.7K transfectants was unmodified. Our findings indicate that the adenovirus 14.7K protein functions as a specific inhibitor of TNF cytolysis in the absence of other adenovirus proteins and thus is a unique tool to study the mechanism of TNF cytotoxicity.

Tumor necrosis factor (TNF), an immunomodulator produced by macrophages and lymphocytes, is responsible for a wide variety of physiological effects. Originally discovered to cause hemorrhagic necrosis of murine sarcomas (11), TNF is now known to suppress the production of lipoprotein lipase in adipocytes (4, 74), induce acute-phase proteins in the liver (49), and mediate lipopolysaccharide-induced shock in mice (3, 75). TNF also has a wide variety of immunologic effects: TNF enhances expression of class I major histocompatibility complex (MHC) molecules (14, 57), induces fibroblast growth (78), increases neutrophil adhesion (23), activates polymorphonuclear leukocytes (67, 68), augments natural killer cell cytotoxicity (51), and stimulates B-cell differentiation (37).

The antitumor and antiviral effects of TNF have been well established. TNF is cytotoxic to a wide variety of primary tumors and transformed cell lines (8, 22, 61, 72), although not all malignant cells are sensitive to TNF (41, 72, 76). TNF can also suppress virus replication in cells infected with either RNA or DNA viruses (35, 40, 48, 83, 85). TNF can lyse cells infected with vesicular stomatitis virus, herpes simplex virus type 2, or adenovirus (2, 48, 83). TNF can also inhibit B-cell activation by Epstein-Barr virus (36) and prevent infection by human immunodeficiency virus (85).

The mechanism of TNF-induced cytolysis remains obscure. Although TNF induces many cellular factors, including superoxide dismutase (82, 84); prostaglandin E_2 (43); colony-stimulating factors (39, 50); interleukins 1, 6, and 8 (16, 17, 39, 46, 71); the epidermal growth factor receptor (53); and the interleukin 2 receptor (42, 44, 63), it is not clear which if any of these molecules is involved in the TNF-induced lytic process.

Adenovirus-infected mouse C3HA fibroblasts are made sensitive to TNF cytolysis by expression of the adenovirus E1A genes (12). E1A-expressing cell lines are often (12, 18), although not always (26), spontaneously sensitive to TNF cytolysis. TNF cytolysis of adenovirus-infected C3HA cells is suppressed, however, when the E3 14,700-kDa protein (14.7K) encoded by the adenovirus E3 transcription unit is expressed in the infected cell (26). The 14.7K protein is a general mediator of TNF suppression; it can suppress cytotoxicity in cells spontaneously sensitive to TNF and in resistant cells made sensitive to TNF by inhibitors of protein or RNA synthesis or by treatment of cells with cytochalasin E (cytE) (27).

Our previous studies were carried out in cells infected by wild-type adenovirus or mutants lacking the 14.7K and other E3 genes (26, 27). Although these studies established that 14.7K is necessary to prevent TNF cytolysis, the studies did not establish that 14.7K is sufficient. We could not eliminate the possibility that other adenovirus proteins, coded within regions of adenovirus for which deletion mutants are not available, act with 14.7K to mediate suppression of TNF

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lysis. To determine whether 14.7K functions independently of other adenovirus proteins, we have isolated permanently transfected C3HA and C127 cell lines expressing the 14.7K protein. These transfectants were then tested for sensitivity to TNF cytolysis. Our results indicate that 14.7K can indeed protect cells against TNF cytolysis in the absence of other adenovirus proteins.

MATERIALS AND METHODS

Cell lines and viruses. C3HA, a mouse embryo fibroblast (25), and C127, a fibroblastlike line derived from a mouse mammary tumor (19), were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum (growth media). KB human oral carcinoma cells were used to grow wild-type adenoviruses and adenovirus E3 deletion mutants. Virus titers were determined by plaque assay on A549 human lung carcinoma cells.

Group C adenovirus type 5 (Ad5) E3 mutants have been described. *rec*700 is an Ad5/2/5 construct in which the Ad5 *Eco*RI C fragment has been replaced by the equivalent Ad2 *Eco*RI D fragment (81). *rec*5/2 *dl*762 deletes nucleotides 2904 to 3251 in the E3 transcription unit of *rec*700, a deletion that removes only the 14.7K gene and leaves other E3 genes intact (5a). *rec*5/2 *dl*706 deletes nucleotides 1604 to 2238; this mutant overproduces mRNA *h*, resulting in an increased expression of the 14.7K protein (5). Both *dl*762 and *dl*706 were derived from *rec*700.

Plasmids and transfections. The 14.7K gene was inserted into a pBR322-derived bovine papillomavirus (BPV) vector, pBMT-3x, which has been described previously (13). This vector contains both the mouse metallothionein gene and the human metallothionein IA gene; the latter confers resistance to the toxic effects of heavy metals and thus functions as a dominant selection marker. This plasmid also contains the 69% *Bam*HI-*Hin*dIII fragment of BPV type I (BPV-1) that includes the open reading frames E1 through E8 (54). The 14.7K sequence was removed from pKA-14.7K E (59a) with *Xho*I. This segment, which includes nucleotides 2880 to 3382 in the E3 transcription unit of *rec*700, contains the entire 14.7K gene without the E3 promoter. The 14.7K fragment was ligated into the *Xho*I site within the first exon of the mouse metallothionein gene, putting the 14.7K gene under control of the mouse metallothionein promoter. The 14.7K vector, designated pBMT-14.7K, was found to express constitutively the 14.7K protein; expression of 14.7K could be further induced with 20 to 50 μM $CdCl_2$.

C3HA fibroblasts were transfected by using lipofectin reagent (Bethesda Research Laboratories, Bethesda, Md.), and stable transfectants were selected with 10 μM $CdCl_2$. Fifteen micrograms of pBMT-3x or pBMT-14.7K was combined with an equal volume of lipofectin and added to 5×10^5 cells seeded overnight on 60-mm plates. After a 24-h incubation, the cells were treated with 3 ml of 15% glycerol-BES [*N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; Calbiochem, La Jolla, Calif.] in serum-free DMEM for 2 min. The cells were incubated for 48 h at 37°C in 8% CO_2, removed with trypsin, and split 1:3 into DMEM supplemented with 10% fetal calf serum (FCS) and 10 μM $CdCl_2$. Cells were refed every 3 days until discrete colonies appeared; the colonies were harvested with cloning cylinders. Transfectants containing the pBMT-3X plasmid are designated C3HA/pBMT, and transfectants containing the pBMT-14.7K plasmid are designated C3HA/p14.7K.

C127 cells were seeded as described above and transfected by using calcium phosphate DNA coprecipitation as previously described (28). After a 4-h incubation, the cells were treated with 15% glycerol-BES in serum-free DMEM for 1 min. After 48 h, stable transfectants were selected as described above by using 20 μM $CdCl_2$.

Hybridoma production, cloning, and ascites preparation. A TrpE-14.7K fusion protein and TrpE protein were prepared in *Escherichia coli* and purified as previously described (73). Production of monoclonal antibodies (MAbs) against TrpE-14.7K was conducted at the hybridoma facility at the University of Georgia under the direction of Lee H. Pratt. Briefly, female BALB/c mice were given intraperitoneal injections of 100 μg of TrpE-14.7K in 0.2 ml of complete Freund's adjuvant. After 14 days, each mouse was boosted with 50 μg of fusion protein in 0.05 ml of incomplete Freund's adjuvant. Three days after boosting, spleen cells from the immunized mice were fused with SP2/0-Ag14 myeloma cells by using polyethylene glycol (Boehringer Mannheim, Indianapolis, Ind.) and cultured as previously described (15). Hybridoma culture fluids were screened against TrpE-14.7K and TrpE by a standard enzyme-linked immunosorbent assay (ELISA) (58). Positive clones were rescreened against the fusion protein and against TrpE. Antibodies that reacted against both proteins were specific for TrpE. Antibodies that reacted with only the fusion protein were positive for 14.7K and were recloned by limiting dilution. Ascites fluid was prepared in pristane-primed BALB/c mice.

Purification, characterization, and biotinylation of MAbs. MAbs to 14.7K (MAb 3 and MAb 5) were purified from ascites fluid by affinity chromatography with a protein G Sepharose 4FF column (Pharmacia, Piscataway, N.J.). Immunoglobulin isotypes were identified by using isotype-specific mouse polyclonal antibodies (Meloy Laboratories, Springfield, Va.) in an Ouchterlony double-immunodiffusion assay. NHS-LC-BIOTIN (Pierce Chemicals, Rockford, Ill.) was used to biotinylate proteins (1 mg/ml) essentially according to manufacturer's instructions by using a reagent-to-protein molar ratio of 20:1. Unreacted biotin was removed by dialysis at 4°C against 0.02 M phosphate buffer and 0.02% sodium azide (NaN_3), pH 7.4, for 72 h.

Virus infections and lysate preparation for immunoblots. C3HA and C127 cells were infected with *rec*700 or E3 14.7K mutants at multiplicities of infection ranging from 30 to 200 PFU per cell. After 24 h, the cells were removed with trypsin and lysed with 1× Laemmli sample buffer (10% glycerol, 2% [wt/vol] sodium dodecyl sulfate [SDS], 60 mM Tris [pH 6.8]). Infection rates were determined in each experiment by indirect immunofluorescence on fixed monolayers by using an antiserum against Ad5 (American Type Culture Collection, Rockville, Md.). Monolayers were then incubated with a fluorescein-conjugated goat anti-rabbit antiserum (Meloy), and the fraction of cells infected was determined by fluorescence microscopy. Cells were fixed 48 h after infection. For all reported experiments 90 to 100% of cells were infected.

Transfected-cell lysates and polyacrylamide gel electrophoresis. Transfected cells were grown to confluency on 100-mm plates in DMEM or DMEM supplemented with 5 to 50 μM $CdCl_2$ (as indicated) for 24 h and then lysed as described above. Cell lysates were removed with a rubber policeman. Protein concentrations were determined by using bicinchoninic acid as previously described (69). β-Mercaptoethanol (1% final concentration) was added to boiled-cell lysates, and samples were then electrophoresed on an SDS–12% polyacrylamide gel electrophoresis minigel (Bio-Rad, Richmond, Calif.). Proteins were loaded at 20 to 32 μg per lane, as indicated.

Immunoblots. Proteins were transferred to a hybridization transfer membrane (Micron Separations, Westboro, Mass.) in transfer buffer (48 mM Tris [pH 9.2], 39 mM glycine, 1.3 mM SDS, 20% methanol) by using a semidry blotter (Bio-Rad). The membrane was incubated overnight at 4°C in blocking buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris [pH 7.4], 0.02% NaN_3, 0.05% Nonidet P-40, 0.25% gelatin, 5% Carnation nonfat dry milk, 100 U of heparin per ml).

The membrane was incubated in a heat-sealable pouch (Kapak, Minneapolis, Minn.) for 2 h with a 14.7K polyclonal antiserum (1:400) (73) or a 14.7K MAb (1:1,000) in TTBS (500 mM NaCl, 20 mM Tris [pH 7.5; TBS] with 0.05% Tween 20 and 1% gelatin). Membranes incubated with MAbs were washed twice in blocking buffer and then incubated with rabbit immunoglobulin anti-mouse antibody (1:100) (Fisher Scientific, Orangeburg, N.Y.) for 2 h. All membranes were then washed twice in blocking buffer and incubated with 5 μCi of ^{125}I-protein A (specific activity, 30 μCi/μg) (ICN Biochemicals, Westboro, Mass.) in 150 mM NaCl–25 mM Tris (pH 7.5)–25 mg of bovine serum albumin (BSA) per ml–0.02% NaN_3. After 1 h, the membranes were washed four times in TTBS, once in TBS, and once in distilled water. The blots were air dried and exposed for 14 to 36 h as indicated. ^{14}C-labeled molecular mass markers were purchased from Amersham, Arlington Heights, Ill.

ELISA. Cell lysates to quantitate 14.7K were prepared as previously described (73) except that the lysates were rapidly frozen and thawed four times in lysis buffer containing 0.4 M NaCl. A sandwich ELISA system was developed by the method of Pratt et al. (58). All incubations were performed at room temperature for 2 h unless otherwise indicated.

Microtiter plates (Immulon 4; Dynatech, Alexandria, Va.) were coated with 50 μl of MAb 5 (10 μg/ml) and washed three times with wash buffer (10 mM Tris-HCl, 0.05% Tween 20, 0.02% NaN_3 [pH 8.0]), and nonspecific protein binding was blocked with 50 μl of 2% BSA in phosphate-buffered saline (PBS) for 30 min. The plates were washed, and 50 μl of diluted lysates or of TrpE-14.7K fusion protein was added. After being washed, the plates were incubated with 50 μl of biotin-labeled MAb 3 (10 μg/ml), washed, and then incubated with 50 μl of a 1:10,000 dilution of ExtrAvidin alkaline phosphatase (Sigma, St. Louis, Mo.).

Bound alkaline phosphatase was detected by using a two-step amplification system (38). Fifty microliters of NADP in 0.53% diethanolamine–1.0 mM $MgCl_2$ (pH 9.5) was added to the plates and incubated for 30 min. The color was developed by the addition of 100 μl of 0.55 mM *p*-iodonitrotetrazolium violet, 0.125 mg of diaphorase per ml, and 0.0125 mg of alcohol dehydrogenase per ml in 25 mM sodium phosphate (pH 7.2). After a 10-min incubation, the reaction was terminated with 50 μl of 0.4 N HCl and the A_{492} was read with a Titertek multiscan plate reader (Flow Laboratories, McLean, Va.). Background absorbance was determined by using the ELISA in the absence of the 14.7K protein.

TNF cytolysis assay. Cells at a density of 7.5×10^5 were infected with 100 to 150 PFU per cell of wild-type or E3 mutant adenovirus as indicated. The TNF assay was performed as previously described (26). Recombinant human TNF (2.4×10^7 U/mg) was generously supplied by Cetus Corp., Emeryville, Calif. When indicated, cycloheximide (CHX) was added to a final concentration of 20 μg/ml, cytE was added to a final concentration of 2 μg/ml, and $CdCl_2$ was added to a final concentration of 5 to 50 μM. Cells were incubated with TNF for 16 h (with CHX or cytE) or 18 h (without CHX or cytE) at 37°C and 8% CO_2. Results are expressed as percent specific ^{51}Cr release and are determined by using the following formula: percent specific release = (experimental release − control release)/(maximum release − control release) × 100.

Control values were measured as the percent ^{51}Cr release from cells incubated with medium alone or with CHX, cytE, and/or $CdCl_2$ (as indicated) in the absence of TNF. Spontaneous release was <35% at all TNF concentrations. Maximum ^{51}Cr release was determined by lysing cells with 100 μl of 1 N HCl prior to harvest. Infection rates were determined by indirect immunofluorescence as described above.

Iodination of TNF and direct radioligand-binding assay. Purified human recombinant TNF (12 μg) was radioiodinated with $Na^{125}I$ by using the Iodogen method (45) to a specific activity of 5.4×10^7 cpm/μg. A direct-binding assay was performed essentially as described by Andrews et al. (1). Briefly, graded amounts of ^{125}I-radioactive TNF were added to C3HA (2.8×10^6 cells), C3HA/pBMT (2.35×10^6 cells), or C3HA/p14.7K (3×10^6 cells) fibroblasts in 1.0 ml of binding buffer (Hanks balanced salt solution [pH 7.2] supplemented with 5% FCS and 0.1% NaN_3) for 1.5 h at 4°C with constant mixing. Nonspecific binding was estimated by the inclusion of a 100-fold excess of nonradioactive TNF in a parallel set of samples. The amount of radioactivity bound was determined by counting the washed pellets in duplicate samples by using a gamma spectrophotometer (Packard B5005). The equilibrium dissociation constant (K_d) and the number of receptors per cell were calculated by the method of Priore and Rosenthal (59).

Flow cytometry analysis. C3HA transfectants were incubated with TNF (final concentration, 500 U/ml) in growth medium for 96 h and then harvested by using 0.02% EDTA in PBS. One million cells were resuspended in 50 μl (80 μg/ml) of anti-class I MHC antibody 16.1.2 and incubated on ice for 45 min. Equivalent numbers of cells were also incubated with a mouse anti-immunoglobulin G2a (80 μg/ml) (Sigma) or anti-transferrin receptor antibody R17 217.1.3 (American Type Culture Collection). After the cells were washed three times in 2 ml of growth medium supplemented with 0.01% NaN_3, the cells were resuspended in 50 μl of goat anti-mouse immunoglobulin phycoerythrin conjugate (1:10; Fisher Scientific) or fluorescein isothiocyanate conjugate (1:20; Fisher Scientific) for 45 min. Cells were washed and resuspended in 1% paraformaldehyde (Sigma) in PBS. Flow cytometry was performed by using a FACScan (Becton Dickinson, Sunnyvale, Calif.). All samples incubated with anti-immunoglobulin G2a displayed a mean fluorescence intensity of <5 U.

RESULTS

The 14.7K protein is expressed in C3HA and C127 cells stably transfected with the 14.7K gene. The entire Ad5 14.7K gene was inserted into a BPV vector (pBMT-14.7K) under control of the mouse metallothionein promoter. The plasmid was then stably transfected into C3HA and C127 cells. Multiple transfected clones from each cell line were tested for expression of the 14.7K protein, and a representative clone from each transfection was chosen for study.

Figure 1 shows a Western immunoblot with a polyclonal antibody against a TrpE-14.7K fusion protein. This immunoblot shows that C3HA (Fig. 1A, lane 3) and C127 (Fig. 1B, lane 3) cells transfected with pBMT-14.7K express the 14.7K protein. C3HA cells transfected with the parent plasmid not containing the 14.7K gene (C3HA/pBMT) did not express

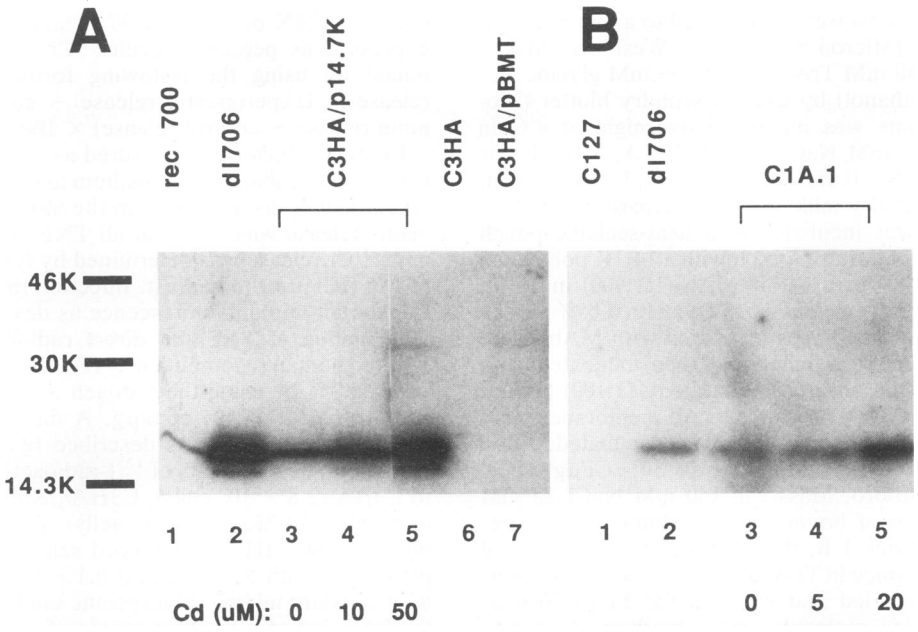

FIG. 1. Immunoblots of C3HA and C127 cells transfected with 14.7K. (A) C3HA cells were uninfected (lane 6) or infected for 24 h with either the wild-type adenovirus *rec*700 (lane 1) or the E3 deletion mutant *dl*706, which overproduces 14.7K (lane 2). C3HA/pBMT transfectants (not expressing 14.7K) (lane 7) and C3HA/p14.7K transfectants (expressing 14.7K) (lane 3) were left uninduced or induced with $CdCl_2$ for 24 h (lanes 4 and 5, respectively) before lysates were made (see Materials and Methods). (B) C127 cells were left uninfected (lane 1), infected with *dl*706 (lane 2), or transfected with pBMT-14.7K (C1A.1, lanes 3 to 5). C1A.1 cells were exposed to various concentrations of $CdCl_2$ as indicated. Lysates were incubated with a polyclonal antiserum against a TrpE-14.7K protein (73). ^{14}C-molecular mass markers are indicated. Twenty micrograms of protein per lane was loaded. The autoradiogram was exposed for 36 h.

any protein cross-reacting with the 14.7K antiserum (Fig. 1A, lane 7). The 14.7K protein often appears as a triplicate in immunoblots (73).

The 14.7K gene is under control of the mouse metallothionein promoter, which can be induced by divalent cations such as cadmium (13, 54). The amount of 14.7K produced by C3HA/p14.7K could be increased with 50 μM $CdCl_2$ (Fig. 1A, lane 5), although 14.7K was only slightly induced with 10 μM $CdCl_2$ (Fig. 1A, lane 4). C127 transfectants also made 14.7K constitutively, and 20 μM $CdCl_2$ increased the amount of 14.7K produced (Fig. 1B, lanes 3 to 5).

The 14.7K transfectants produced a significant amount of 14.7K compared with that produced by cells infected with adenovirus. The 14.7K transfectants expressed more 14.7K than cells infected with the wild-type virus *rec*700 (Fig. 1A, lane 1). The C127 14.7K transfectant produced similar amounts of 14.7K as cells infected with *dl*706, which overproduces the 14.7K protein (Fig. 1B, lane 2 and 3). Similarly, the C3HA/p14.7K cells induced with 50 μM $CdCl_2$ expressed similar amounts of 14.7K, as did C3HA cells infected with *dl*706 (Fig. 1A, lanes 2 and 5). In either transfection or infection, C3HA transfectants produced more 14.7K than C127 cells. These results were confirmed by ELISA analysis (see below and Table 1).

Quantitation of 14.7K in transfected and infected cells. Having determined that 14.7K is expressed in cells transfected with the 14.7K gene, we next wanted to quantitate more precisely the amount of 14.7K produced in transfected cells. A sandwich ELISA to quantitate the amount of 14.7K produced by the C3HA and C127 14.7K transfectants was developed. Two MAbs specific for different 14.7K epitopes within a TrpE-14.7K fusion protein were produced. Figure 2B shows that MAb 3 and MAb 5 bound to 14.7K extracted from C3HA cells infected with *rec*700 (lane 4) and also from the C3HA/p14.7K transfectants (lane 2); the combination of these two MAbs reacted with intensities as strong as the TrpE-14.7K polyclonal antiserum (Fig. 2A). A MAb against TrpE did not bind to 14.7K under similar conditions (data not shown).

The sandwich ELISA consisted of MAb 5 bound to the solid phase, antigen, and biotin-labeled MAb 3. The amount of labeled MAb 3 bound to antigen was amplified by using a two-step amplification system (see Materials and Methods) and quantitated by spectrophotometry (A_{492}). The amount of 14.7K in each experiment was determined from a standard curve; absorbance was linear from 0.2 to 4.0 ng per well of the TrpE-14.7K protein. This quantitation is based on the assumption that the antibodies bind equally well to the native 14.7K and the TrpE-14.7K fusion protein.

As measured by ELISA (Table 1), C3HA cells infected with *dl*706 produced about 20-fold more 14.7K than cells infected with *rec*700. Cells infected with *dl*762, which deletes nearly the entire 14.7K gene, did not produce detectable levels of 14.7K. These data are consistent with the 14.7K immunoblot (Fig. 1A, lanes 1 and 2), which showed that C3HA cells infected with *dl*706 produced more 14.7K than cells infected with *rec*700. C3HA/p14.7K also expressed 14.7K, and treatment of C3HA/p14.7K with 20 μM $CdCl_2$ resulted in approximately a 10-fold increase in 14.7K production. These data correlate with the immunoblot which showed 14.7K induction with cadmium in C3HA/p14.7K (Fig. 1A, lane 5).

C127 cells infected with *dl*706, but not *dl*762, produced 14.7K (Table 1). The C127 14.7K transfectant C1A.1 expressed similar levels of 14.7K, as did C127 cells infected with *dl*706. Cadmium induction of 14.7K in C1A.1 resulted in

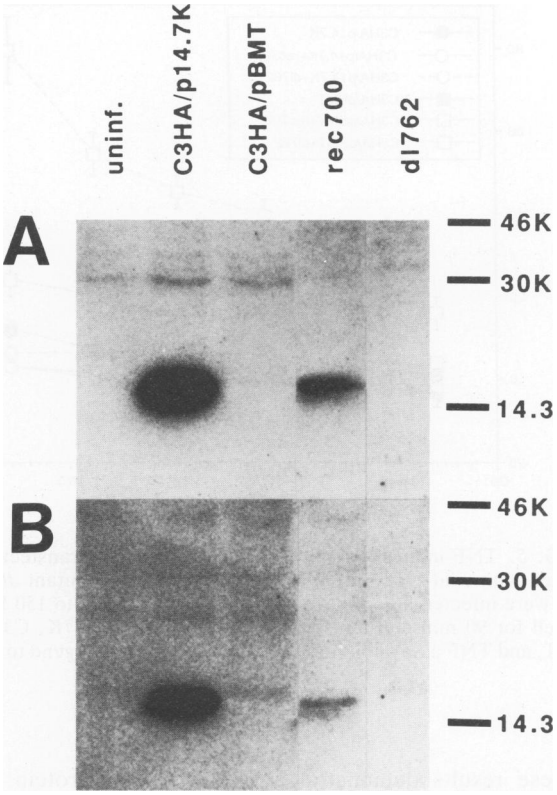

FIG. 2. Immunoblots of C3HA cells transfected with 14.7K antibodies. Parallel immunoblots were incubated with either a TrpE-14.7K polyclonal antiserum (A) (73), or MAb 3 and MAb 5 (B) (see Materials and Methods). C3HA cells (uninf.) were transfected with either pBMT-14.7K (C3HA/p14.7K) or pBMT (C3HA/pBMT). C3HA cells were also infected with either the wild-type adenovirus *rec*700 or the 14.7K deletion mutant *dl*762. Thirty-two micrograms of protein per lane was loaded, and the autoradiograms were exposed for 14 h.

a twofold increase in 14.7K expression (Table 1), which is consistent with that detected by immunoblot (Fig. 1B). Both ELISA analysis (Table 1) and immunoblot examination (Fig. 1) showed that C3HA cells expressed more 14.7K than C127

TABLE 1. Quantitation of 14.7K in cell lysates by ELISA

Cell line	Dilution	A_{492}[a]	14.7K (μg/10^6 cells)[b]
C3HA	1:4	0.049 ± 0.000	—
C3HA + *rec*700	1:100	0.172 ± 0.004	0.238
C3HA + *dl*706	1:1,000	0.468 ± 0.030	5.82
C3HA + *dl*762	1:4	0.037 ± 0.000	—
C3HA/pBMT	1:4	0.045 ± 0.006	—
C3HA/p14.7K	1:20	0.220 ± 0.000	0.786
C3HA/p14.7K + 20 μM $CdCl_2$	1:100	0.343 ± 0.000	7.54
C127	1:4	0.016 ± 0.004	—
C127 + *dl*706	1:50	0.209 ± 0.005	0.290
C127 + *dl*762	1:4	0.036 ± 0.008	—
C1A.1	1:100	0.162 ± 0.006	0.342
C1A.1 + 20 μM $CdCl_2$	1:100	0.160 ± 0.030	0.657

[a] Values represent mean A_{492} minus background absorbance ± standard error of the mean.
[b] —, Negligible.

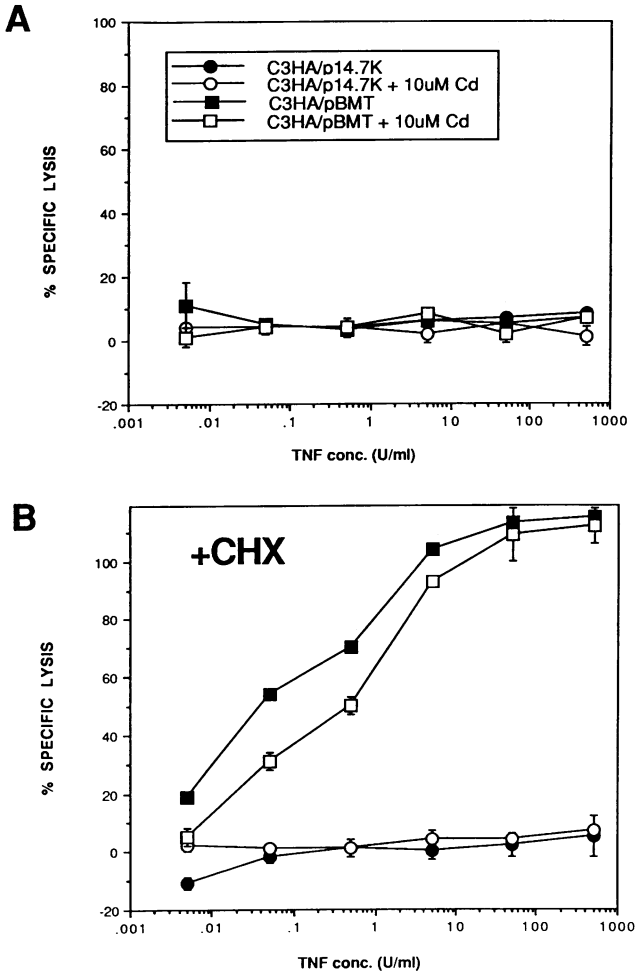

FIG. 3. TNF cytolysis of C3HA fibroblasts transfected with the 14.7K gene. C3HA/p14.7K cells are C3HA fibroblasts stably transfected with pBMT-14.7K. C3HA/pBMT cells are C3HA cells stably transfected with pBMT-3x, which does not produce 14.7K (see Materials and Methods). Representative clones are shown; similar experiments were performed with at least two other clones from each plasmid. Cells were plated at 7.5×10^5 cells per 100-mm plate and labeled 18 h later with 200 μCi of $Na_2{}^{51}CrO_4$. TNF was added 4 h after labeling in the absence (A) or presence (B) of 20 μg of CHX per ml. $CdCl_2$ was added at the same time as TNF. Each point is the average of triplicates. Cytolysis was measured as ^{51}Cr release after a 16 h (B) or 18 h (A) incubation.

cells by either transfection or infection. These data confirm that both the C3HA and C127 14.7K transfectants produce 14.7K and that the amount of 14.7K produced can be induced with cadmium.

14.7K suppresses TNF cytolysis in transfected C3HA and C127 cells treated with CHX. The studies described above suggest that if 14.7K functions in the absence of other adenovirus proteins to suppress TNF cytolysis, then the amounts of protein expressed in transfected cells should be sufficient to protect cells from TNF lysis. C3HA cells are normally resistant to the cytolytic effects of TNF but can be made sensitive to TNF in the presence of protein synthesis inhibitors such as CHX (26). C3HA/pBMT transfectants (not expressing 14.7K) were resistant to TNF (Fig. 3A) but became sensitive to TNF in the presence of 20 μg of CHX

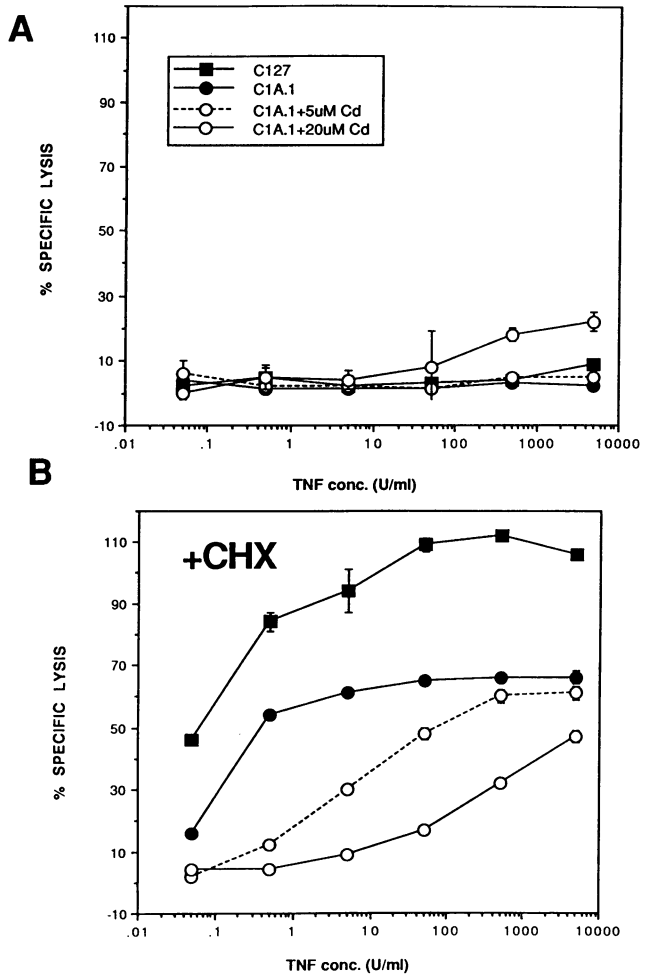

FIG. 4. TNF cytolysis of 14.7K-transfected C127 cells. C1A.1 is a representative clone of C127 cells stably transfected with pBMT-14.7K (see Materials and Methods). TNF assay conditions are described in the legend to Fig. 3.

per ml (Fig. 3B); specific lysis was 100% with 50 U of TNF per ml. TNF susceptibility in the C3HA/pBMT transfectants was not significantly altered by 10 μM $CdCl_2$. C3HA/p14.7K cells expressing 14.7K also were not lysed by TNF in the presence or absence of 10 μM $CdCl_2$ (Fig. 3A); however, in marked contrast to C3HA/pBMT cells, C3HA/p14.7K transfectants were not lysed by TNF in the presence of CHX (Fig. 3B). TNF cytolysis was not significantly altered in the C3HA transfectants by 10 μM $CdCl_2$ (Fig. 3B) or higher cadmium concentrations (20 to 50 μM; data not shown). This is expected, considering that the constitutive level of 14.7K expression was high in these cells (Fig. 1A; Table 1).

As with C3HA cells, C127 cells or C127 14.7K transfectants were not lysed by TNF (Fig. 4A). Also, C127 cells were lysed by TNF in the presence of CHX (Fig. 4B). TNF lysis in the presence of CHX was partially suppressed in the C127 14.7K transfectant (C1A.1) in the absence of cadmium; specific lysis decreased from 100 to 60% at 5 U of TNF per ml and did not increase with TNF concentrations of up to 500 U/ml. Induction of 14.7K with cadmium caused a dose-dependent decrease in TNF sensitivity; specific lysis was reduced from 90 to 10% at 5 U of TNF per ml by using 20 μM $CdCl_2$.

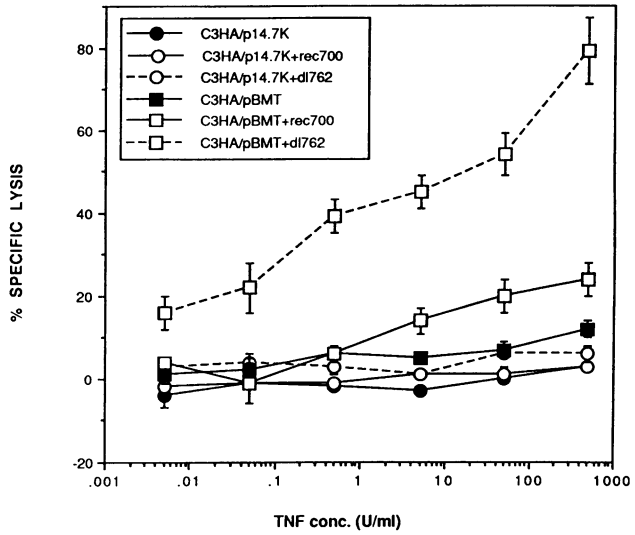

FIG. 5. TNF-induced cytolysis of C3HA 14.7K transfectants infected with wild-type *rec*700 or the 14.7K deletion mutant *dl*762. Cells were infected at a multiplicity of infection of 100 to 150 PFU per cell for 90 min and labeled 18 h later. C3HA/p14.7K, C3HA/pBMT, and TNF assay conditions are described in the legend to Fig. 3.

These results demonstrate that the 14.7K protein can suppress TNF cytolysis in the absence of either adenovirus infection or other adenovirus E3 proteins and that, in the case of C127 cells, increases in 14.7K as a result of cadmium induction (Fig. 1) correlate with a decreased sensitivity to TNF cytolysis. It is interesting to note that while 14.7K provided full protection from TNF cytolysis in C3HA cells, it provided only partial protection in C127 cells in the absence of cadmium induction. This may be a quantitative effect, since C3HA cells produce about twofold more 14.7K than C127 cells (Fig. 1; Table 1). The increased protection observed in the C127 14.7K transfectants following cadmium induction (Fig. 4B) could by due to the approximately twofold increase in the level of 14.7K expression (Table 1). However, it is possible that cadmium induces some other response that synergizes with 14.7K in providing protection against TNF lysis of C127 cells.

14.7K inhibits TNF sensitivity induced by the E1A region of adenovirus. Because 14.7K can suppress TNF sensitivity in cells treated with CHX, we would predict that 14.7K can suppress TNF lysis in cells made sensitive to TNF by other means. In common with protein synthesis inhibitors, the E1A region of adenovirus can sensitize cells to TNF. Cells infected with wild-type adenovirus are resistant to TNF; however, cells infected with adenovirus mutants that delete the E3 14.7K gene are sensitive to TNF cytolysis (26).

Figure 5 shows that the 14.7K protein can suppress TNF sensitivity induced by the adenovirus E1A region. As with C3HA, C3HA/pBMT cells were resistant to TNF when infected with the wild-type adenovirus *rec*700 but were susceptible to TNF lysis when infected with the 14.7K mutant *dl*762. In contrast, C3HA/p14.7K cells were resistant to TNF when infected with either *rec*700 or *dl*762. This indicates that the 14.7K expressed in C3HA/p14.7K cells can function in *trans* to suppress TNF cytolysis in cells infected with adenovirus mutants that lack the 14.7K gene.

14.7K inhibits TNF cytolysis in C3HA cells treated with

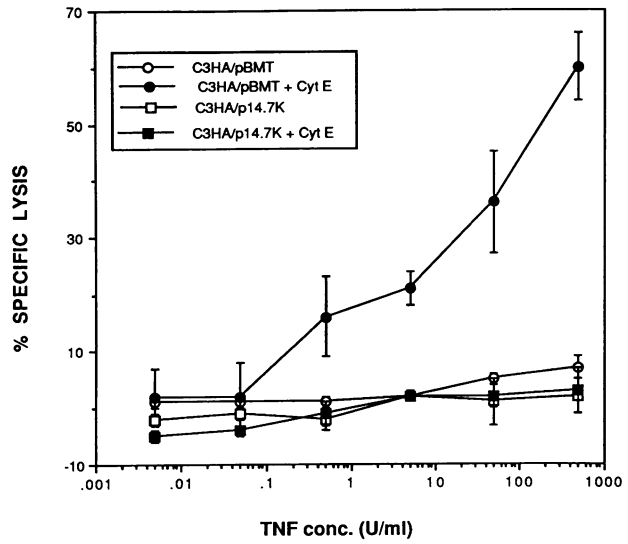

FIG. 6. TNF-induced cytolysis of C3HA 14.7K transfectants treated with cytE. Transfectants were incubated with TNF in the absence or presence of 2 μg of cytE per ml as indicated. Cytolysis was measured as ^{51}Cr release after a 16-h (plus cytE) or 18-h (without cytE) incubation. C3HA/pBMT, C3HA/p14.7K, and TNF assay conditions are described in the legend to Fig. 3.

cytE. One of the first events that occur after TNF exposure in cells that will ultimately die from TNF exposure is the dissolution of microfilaments (62). cytE, which disrupts microfilaments, makes C3HA cells sensitive to TNF (27). The 14.7K protein protects against TNF cytolysis induced by CHX, and one might anticipate that the 14.7K protein could block TNF cytolysis induced by cytE. C3HA/pBMT cells not containing the 14.7K gene were susceptible to TNF cytolysis in the presence of 2 μg of cytE per ml (Fig. 6); approximately 60% of the cells were lysed in the presence of 500 U of TNF per ml. In contrast, C3HA/p14.7K cells were resistant to the effects of TNF in the presence of cytE. This demonstrates that the 14.7K protein can protect C3HA cells from TNF cytolysis in the presence of cytE.

14.7K does not act by altering TNF receptor density or affinity in C3HA fibroblasts. The C3HA 14.7K transfectants were used to investigate the mechanism of 14.7K-induced suppression of TNF cytolysis. One hypothesis is that 14.7K can alter TNF receptor density or affinity. An ^{125}I-TNF direct radioligand-binding assay was used to determine TNF receptor characteristics in the parent C3HA cell line and in the C3HA transfectants. Scatchard analysis of direct-binding data obtained at 4°C yielded a straight line for the parent C3HA cell line, the C3HA/pBMT cells, and the C3HA/p14.7K transfectants, which is consistent with a single class of radioactive TNF-binding sites. The K_d and number of binding sites per cell were not significantly different between C3HA fibroblasts and the C3HA 14.7K transfectants ($P > 0.05$) (Table 2). These data indicate that the 14.7K protein does not alter either the TNF receptor affinity or the cell surface receptor number in C3HA fibroblasts. These data also imply that 14.7K does not inhibit TNF cytolysis by altering TNF receptor characteristics in this cell line.

14.7K does not alter class I MHC induction. TNF has previously been shown to increase cell surface expression of class I MHC molecules on several cell types, including endothelial cells and fibroblasts (14, 57). Increases in steady-state mRNA levels result in augmented cell surface class I MHC antigens (14, 57), although posttranscriptional regulation may also be involved in some cell lines (57). To induce cell surface class I MHC antigens, an intracellular signal from the TNF receptor must successfully produce the intracellular modifications necessary to increase class I MHC expression. TNF induction of class I MHC molecules, therefore, was used to determine if this intracellular signalling pathway is intact in 14.7K transfectants. C3HA fibroblasts constitutively express H-2K^k class I MHC molecules and, if 14.7K acts by interrupting general signal transduction pathways induced by TNF, we would predict that 14.7K would alter TNF induction of class I MHC expression. C3HA transfectants were grown with 500 U of TNF per ml for 96 h, and class I MHC levels were determined by flow cytometry. TNF induced C3HA class I MHC molecules, and the 14.7K protein did not affect the level of induction (Table 3). TNF induction was specific for class I MHC molecules; TNF did not increase cell surface expression of transferrin receptors (data not shown). TNF (500 U/ml) increased class I MHC levels approximately twofold in C3HA fibroblasts, which is in agreement with previous reports of TNF-induced class I MHC expression on fibroblasts (14). This experiment indicates that the 14.7K protein leaves at least one general intracellular signalling system intact. These findings suggest two possible modes of 14.7K action. 14.7K may act only on the signalling pathways required for the cytolytic function of TNF. Alternatively, 14.7K may act downstream of signalling on the lytic processes themselves.

TABLE 2. Characterization of TNF receptors by Scatchard analysis[a]

Cell line	K_d (nM)	Receptor no./cell
C3HA	5.5 ± 1.8	11,800 ± 1,640
C3HA/pBMT	3.1 ± 0.31	18,100 ± 1,510
C3HA/p14.7K	3.3 ± 0.68	9,850 ± 1,690

[a] Values are reported as means ± standard errors of the mean.

DISCUSSION

TNF lyses a wide variety of tumor- and virus-infected cells. Although the mechanism of TNF cytolysis is unknown, TNF induces the synthesis of a wide variety of cellular molecules, some of which have been implicated in

TABLE 3. FACS analysis of cell surface class I MHC molecules in C3HA 14.7K transfectants

Expt no. and cell line	Mean fluorescence intensity[a]		Induction (fold)
	−TNF	+TNF	
1[b]			
C3HA/pBMT	32	90	2.8
C3HA/p14.7K	58	140	2.4
2[c]			
C3HA/pBMT	290	750	2.6
C3HA/p14.7K	550	900	1.6

[a] Fluorescence intensity was measured as forward light scatter defined in arbitrary linear units.
[b] Antibodies were detected with fluorescein isothiocyanate conjugate.
[c] Antibodies were detected with phycoerythrin conjugate.

pathogenesis (47, 82). Specific inhibitors of the TNF-induced lytic process could be used to examine the mechanism of TNF cytolysis. Several TNF-binding proteins have been isolated from urine and serum of cancer patients, and some of these proteins are homologous to the TNF receptor and are able to inhibit ^{125}I-TNF binding (20, 21, 55, 66). The adenovirus E3 14.7K protein, however, is the only known intracellular inhibitor of TNF and thus is a unique tool for examining the mechanism of TNF cytolysis. Furthermore, results reported here indicate that the 14.7K protein may be a specific inhibitor of the intracellular processes involved in the TNF-induced lytic mechanism.

In this paper we have isolated and examined two different transfected murine cell lines that express 14.7K, and we have demonstrated that 14.7K can suppress TNF cytolysis in the absence of other adenovirus proteins. We have also shown, both by ELISA and by immunoblots, that the amount of intracellular 14.7K expressed correlates with the degree of TNF suppression. C3HA cells express more 14.7K than C127 cells when the 14.7K gene is introduced by either infection or transfection.

These latter two observations suggest that 14.7K may function in a stoichiometric rather than a catalytic role. For example, 14.7K could physically interact with a component of the TNF lysis pathway. This notion would be in accord with the emerging consensus that many DNA tumor virus proteins act by binding to preexisting cellular proteins (31, 80). In any case, since it is now clear that 14.7K can function without other adenovirus proteins to prevent TNF cytolysis, these hypotheses may be tested by using transfected cells. Since 14.7K alone can prevent TNF cytolysis of both C3HA and C127 cells, it may be possible to test whether 14.7K functions in the same manner in these cells.

In the experiments reported here, 14.7K was expressed from a BPV vector that includes the open reading frames E1 through E8 (54). These open reading frames encode functions for maintenance of BPV as a multicopy plasmid, for transactivation and transrepression of BPV genes, and for morphological transformation. These BPV proteins by themselves do not influence TNF cytolysis, because cells transfected with the control vector (C3HA/pBMT) were indistinguishable from untransfected C3HA when treated with TNF. However, we cannot exclude the possibility that these BPV proteins potentiate or act in concert with 14.7K to prevent TNF cytolysis.

The mechanism of 14.7K suppression of TNF cytolysis remains unclear, although our evidence suggests that 14.7K acts on an intracellular pathway specific for the TNF-induced lytic process. This is consistent with the subcellular localization of 14.7K to the cytoplasm and nucleus in both infected and transfected cells (27; unpublished data). Our ^{125}-I-TNF-binding data demonstrate that transfection of C3HA fibroblasts with 14.7K does not substantially alter the affinity of the TNF receptor or the receptor number per cell. C3HA fibroblasts have a single class of high-affinity TNF receptors with a dissociation constant of 5 nM and approximately 10,000 receptors per cell. Although transfection with the pBMT plasmid (not containing 14.7K) doubled the receptor number ($P < 0.025$), it is unlikely that this difference would alter receptor function. In any case, the parent C3HA cell line and the C3HA 14.7K transfectant had nearly identical TNF receptor numbers. Two types of TNF receptors have been identified: a 55- and a 75-kDa species, both of which have been associated with different cell types (7, 29, 32, 34, 70, 76). Studies are under way to further characterize the type of TNF receptor found on C3HA fibroblasts.

The relationship among receptor number, receptor affinity, and TNF sensitivity is complex. Watanabe et al. (79) suggested that the precise relationship between receptor number and TNF sensitivity remains uncertain. In many cases TNF responsiveness is not related to the number or affinity of TNF-binding sites, and Schutze et al. (65) have suggested that postreceptor mechanisms control tissue-specific cellular TNF responses.

TNF receptor-ligand signalling pathways also appear to be cell type dependent. TNF intracellular signalling involves multiple second-messenger systems. Lehmmann et al. (43) demonstrated that TNF activates murine peritoneal macrophages through a cyclic AMP-dependent mechanism. Both Brenner et al. (6) and Schutze et al. (65) have shown that TNF can stimulate the activation of protein kinase C, an effect that is cell type dependent (64). Here we show that 14.7K does not inhibit at least one of the general signal transduction pathways induced by TNF, i.e., TNF increases cell surface class I MHC molecules regardless of the presence or absence of the 14.7K protein. It has been previously shown that TNF can stimulate class I MHC molecules by inducing an NF-κB-like molecule (33, 56), an effect that is independent of 14.7K expression. TNF induces class I MHC molecules on C3HA fibroblasts about twofold, as determined by flow cytometry; this level of induction is in agreement with previously reported levels of TNF class I MHC induction in fibroblasts (14).

Because 14.7K does not affect TNF induction of class I MHC molecules, it is possible that 14.7K inhibits only those signal pathways specific for TNF cytotoxicity. This implies that 14.7K may be a specific inhibitor of the TNF-induced lytic process; 14.7K does not interfere in a general manner with the physiological functioning of the cell, and it appears to alter only those pathways restricted to TNF cytolysis. Alternatively, 14.7K may act downstream to suppress the lytic mechanisms themselves.

Although 14.7K has little effect on the ability of TNF to induce the expression of cell surface class I MHC molecules, C3HA cells transfected with the 14.7K gene expressed higher basal levels of class I MHC antigens. C3HA/p14.7K cells reproducibly displayed nearly double the class I MHC antigen fluorescence intensity as C3HA/pBMT cells lacking the 14.7K gene. This increase in class I MHC molecules seen on the surface of the C3HA 14.7K transfectant, however, could be due to clonal variation. Other adenovirus proteins, such as the E3 gp19K protein, alter MHC expression (9, 10, 52, 77), and the decreases in cell surface class I MHC expression caused by gp19K have been postulated to protect virus-infected cells from antivirus cytotoxic T lymphocytes (10, 60). It is, however, unclear how 14.7K-induced increases in class I MHC antigens might alter adenovirus pathogenesis.

As with gp19K, the 14.7K sequence and function are conserved in many human adenovirus serotypes (30). Both proteins may play a role in adenovirus immune evasion in vivo. The E3 14.7K protein may protect virus-infected cells from lysis by TNF, just as the gp19K protein has been postulated to protect cells against lysis by cytotoxic T lymphocytes (26, 30, 60). The significance of TNF in the removal of virus-infected cells in vivo is unknown. However, Ginsberg et al. (24) have shown that intranasal injection of adenovirus E3 virus mutants into cotton rats alters the pulmonary inflammatory response. Injection of *sub*304, which is missing the 14.7K protein (as well as the E3 10.4K and 14.5K proteins) but not the gp19K protein, results in an increased number of neutrophils in a usually monocytic

infiltrate (24). This is consistent with the possibilities that TNF plays a role in removing adenovirus-infected cells in vivo and that 14.7K acts to neutralize this effect of TNF.

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REFERENCES

1. **Andrews, J. S., A. E. Berger, and C. F. Ware.** 1990. Characterization of the receptor for tumor necrosis factor (TNF) and lymphotoxin (LT) on human T lymphocytes. TNF and LT differ in their receptor binding properties and the induction of MHC class I proteins on a human CD4+ T cell hybridoma. J. Immunol. **144**:2582–2591.
2. **Arakawa, T., Y.-R. Hsu, E. Toth, and N. Stebbing.** 1987. The antiviral activity of recombinant human tumor necrosis factor-α. J. Interferon Res. **7**:103–105.
3. **Beutler, B., I. W. Milsark, and A. C. Cerami.** 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science **229**:869–871.
4. **Beutler, B. A., and A. Cerami.** 1985. Recombinant interleukin 1 suppresses lipoprotein lipase activity in 3T3-L1 cells. J. Immunol. **135**:3969–3971.
5. **Bhat, B. M., H. A. Brady, M. H. Pursley, and W. S. M. Wold.** 1986. Deletion mutants that alter differential RNA processing in the E3 complex transcription unit of adenovirus. J. Mol. Biol. **190**:543–557.

5a. **Brady, H., A. Scaria, and W. S. M. Wold.** Unpublished data.

6. **Brenner, D. A., M. O'Hara, P. Angel, M. Chojkier, and M. Karin.** 1989. Prolonged activation of *jun* and collagenase genes by tumour necrosis factor-α. Nature (London) **337**:661–663.
7. **Brockhaus, M., H.-J. Schoenfeld, E.-J. Schlaeger, W. Hunziker, W. Lesslauer, and H. Loetscher.** 1990. Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. Proc. Natl. Acad. Sci. USA **87**:3127–3131.
8. **Browning, J., and A. Ribolini.** 1989. Studies on the differing effects of tumor necrosis factor and lymphotoxin on the growth of several human tumor lines. J. Immunol. **143**:1859–1867.
9. **Burgert, H.-G., and S. Kvist.** 1985. An adenovirus type 2 glycoprotein blocks cell surface expression of human histocompatibility class I antigens. Cell **41**:987–997.
10. **Burgert, H.-G., J. L. Maryanski, and S. Kvist.** 1987. "E3/19K" protein of adenovirus type 2 inhibits lysis of cytolytic T lymphocytes by blocking cell-surface expression of histocompatibility class I antigens. Proc. Natl. Acad. Sci. USA **84**:1356–1360.
11. **Carswell, A. E., L. J. Old, R. L. Kassel, S. Green, N. Fiore, and B. Williamson.** 1975. An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. USA **72**:3666–3670.
12. **Chen, M.-J., B. Holskin, J. Strickler, J. Gorniak, M. A. Clark, P. J. Johnson, M. Mitcho, and D. Shalloway.** 1987. Induction by E1A oncogene expression of cellular susceptibility to lysis by TNF. Nature (London) **330**:581–583.
13. **Cladaras, C., M. Hadzopoulou-Cladaras, B. K. Felber, G. Pavlakis, and V. I. Zannis.** 1987. The molecular basis of a familial apoE deficiency. An acceptor splice site mutation in the third intron of the deficient apoE gene. J. Biol. Chem. **262**:2310–2315.
14. **Collins, T., L. A. Lapierre, W. Fiers, J. L. Strominger, and J. S. Pober.** 1986. Recombinant human tumor necrosis factor increases mRNA levels and surface expression of HLA-A,B antigens in vascular endothelial cells and dermal fibroblasts *in vitro*. Proc. Natl. Acad. Sci. USA **83**:446–450.
15. **Cordonnier, M.-M., C. Smith, H. Greppin, and L. H. Pratt.** 1983. Monoclonal antibodies to phytochromes. Planta **158**:369–376.
16. **Defilippi, P., P. Poupart, J. Tavernier, W. Fiers, and J. Content.** 1987. Induction and regulation of mRNA encoding 26-kDa protein in human cell lines treated with recombinant human tumor necrosis factor. Proc. Natl. Acad. Sci. USA **84**:4557–4561.
17. **Dinarello, C. A., J. G. Cannon, S. M. Wolff, H. A. Bernheim, B. Beutler, A. Cerami, I. S. Figari, M. A. Palladino, Jr., and J. V. O'Connor.** 1986. Tumor necrosis factor (cachectin) is an endogenous pyrogen and induces production of interleukin 1. J. Exp. Med. **163**:1433–1450.
18. **Duerksen-Hughes, P., W. S. M. Wold, and L. R. Gooding.** 1989. Adenovirus E1A renders infected cells sensitive to cytolysis by tumor necrosis factor. J. Immunol. **143**:4193–4200.
19. **Dvoretzky, I., R. Shober, S. K. Chattopadhyay, and D. R. Lowy.** 1980. A quantitative *in vitro* focus assay for bovine papilloma virus. Virology **103**:369–375.
20. **Engelmann, H., D. Aderka, M. Rubinstein, D. Rotman, and D. Wallach.** 1989. A tumor necrosis factor-binding protein purified to homogeneity from human urine protects cells from tumor necrosis factor toxicity. J. Biol. Chem. **264**:11974–11980.
21. **Engelmann, H., D. Novick, and D. Wallach.** 1990. Two tumor necrosis factor-binding proteins purified from human urine. J. Biol. Chem. **265**:1531–1536.
22. **Fransen, L., J. Van der Heyden, R. Ruysschaert, and W. Fiers.** 1986. Recombinant tumor necrosis factor: its effect and its synergism with interferon-gamma on a variety of normal and transformed human cell lines. Eur. J. Cancer Res. Clin. Oncol. **22**:419–426.
23. **Gamble, J. R., J. M. Harlan, S. J. Klebanoff, and M. A. Vadas.** 1985. Stimulation of the adherence of neutrophils to umbilical vein endothelium by human recombinant tumor necrosis factor. Proc. Natl. Acad. Sci. USA **82**:8667–8671.
24. **Ginsberg, H. S., U. Lundholm-Beauchamp, R. L. Horswood, B. Pernis, W. S. M. Wold, R. M. Chanock, and G. A. Prince.** 1989. Role of early region 3 (E3) in pathogenesis of adenovirus disease. Proc. Natl. Acad. Sci. USA **86**:3823–3827.
25. **Gooding, L. R.** 1979. Specificities of killing by T lymphocytes generated against syngeneic SV40 transformants: studies employing recombinants within the H-2 complex. J. Immunol. **122**:1002–1008.
26. **Gooding, L. R., L. W. Elmore, A. E. Tollefson, H. A. Brady, and W. S. M. Wold.** 1988. A 14,700 MW protein from the E3 region of adenovirus inhibits cytolysis by tumor necrosis factor. Cell **53**:341–346.
27. **Gooding, L. R., I. O. Sofola, A. E. Tollefson, P. Duerksen-Hughes, and W. S. M. Wold.** 1990. The adenovirus E3-14.7K protein is a general inhibitor of TNF-mediated cytolysis. J. Immunol. **145**:3080–3086.
28. **Graham, F. L., and A. J. van der Eb.** 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. Virology **52**:456–467.
29. **Hohmann, H.-P., R. Remy, M. Brockhaus, and A. P. G. M. van Loon.** 1989. Two different cell types have different major receptors for human tumor necrosis factor (TNFα). J. Biol. Chem. **264**:14927–14934.
30. **Horton, T. M., A. E. Tollefson, W. S. M. Wold, and L. R. Gooding.** 1990. A protein serologically and functionally related to the group C E3 14,700-kilodalton protein is found in multiple adenovirus serotypes. J. Virol. **64**:1250–1255.
31. **Howe, J. A., J. S. Mymryk, C. Egan, P. E. Branton, and S. T. Bayley.** 1990. Retinoblastoma growth suppressor and a 300-kDa protein appear to regulate cellular DNA synthesis. Proc. Natl. Acad. Sci. USA **87**:5883–5887.
32. **Imamura, K., D. Spriggs, and D. Kufe.** 1987. Expression of tumor necrosis factor receptors on human monocytes and internalization of receptor bound ligand. J. Immunol. **139**:2989–2992.
33. **Israel, A., O. Le Bail, D. Hatat, J. Piette, M. Kieran, F. Logeat, D. Wallach, M. Fellous, and P. Kourilsky.** 1989. TNF stimulates expression of mouse MHC class I genes by inducing an NF kappa B-like enhancer binding activity which displaces constitutive factors. EMBO J. **8**:3793–3800.
34. **Israel, S., T. Hahn, H. Holtmann, and D. Wallach.** 1986. Binding of human TNF-α to high affinity cell surface receptors: effect of

IFN. Immunol. Lett. **12**:217–224.
35. **Jacobsen, H., J. Mestan, S. Mittnacht, and C. W. Dieffenbach.** 1989. Beta interferon subtype 1 induction by tumor necrosis factor. Mol. Cell. Biol. **9**:3037–3042.
36. **Janssen, O., and D. Kabelitz.** 1988. Tumor necrosis factor selectively inhibits activation of human B cells by Epstein-Barr virus. J. Immunol. **140**:125–130.
37. **Jelinek, D. F., and P. E. Lipsky.** 1987. Enhancement of human B cell proliferation and differentiation by tumor necrosis factor-α and interleukin 1. J. Immunol. **139**:2970–2976.
38. **Johannsson, A., and D. L. Bates.** 1988. Amplification by second enzymes, p. 85–106. *In* D. M. Kemeny and S. J. Challacombe (ed.), Elisa and other solid phase immunoassays: techniques and theories. John Wiley & Sons, Inc., New York.
39. **Kaushansky, K., V. C. Broudy, J. M. Harlan, and J. W. Adamson.** 1988. Tumor necrosis factor-α and tumor necrosis factor-β (lymphotoxin) stimulate the production of granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, and IL-1 in vivo. J. Immunol. **141**:3410–3415.
40. **Kohase, M., D. Henriksen-DeStefano, L. T. May, J. Vilcek, and P. B. Sehgal.** 1986. Induction of β_2-interferon by tumor necrosis factor: a homeostatic mechanism in the control of cell proliferation. Cell **45**:659–666.
41. **Kull, F. C., Jr., S. Jacobs, and P. Cuatrecasas.** 1985. Cellular receptor for ^{125}I-labeled tumor necrosis factor: specific binding, affinity labeling, and relationship to sensitivity. Proc. Natl. Acad. Sci. USA **82**:5756–5760.
42. **Lee, J. C., A. Truneh, M. F. Smith, Jr., and K. Y. Tsang.** 1987. Induction of interleukin 2 receptor (TAC) by tumor necrosis factor in YT cells. J. Immunol. **139**:1935–1938.
43. **Lehmmann, V., B. Benninghoff, and W. Droge.** 1988. Tumor necrosis factor-induced activation of peritoneal macrophages is regulated by prostaglandin E_2 and cAMP. J. Immunol. **141**:587–591.
44. **Lowenthal, J. W., D. W. Ballard, E. Bohnlein, and W. C. Greene.** 1989. Tumor necrosis factor α induces proteins that bind specifically to kappa B-like enhancer elements and regulate interleukin 2 receptor α-chain gene expression in primary human T lymphocytes. Proc. Natl. Acad. Sci. USA **86**:2331–2335.
45. **Markwell, M. A. K., and C. F. Fox.** 1978. Surface-specific iodination of membrane proteins of viruses and eucaryotic cells using 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril. Biochemistry **17**:4807–4817.
46. **Matsushima, K., K. Morishita, T. Yoshimura, S. Lavu, Y. Kobayashi, W. Lew, E. Appella, H. F. Kung, E. J. Leonard, and J. J. Oppenheim.** 1988. Molecular cloning of a human monocyte-derived neutrophil chemotactic factor (MDNCF) and the induction of MDNCF mRNA by interleukin 1 and tumor necrosis factor. J. Exp. Med. **167**:1883–1893.
47. **Matthews, N., M. L. Neale, S. K. Jackson, and J. M. Stark.** 1987. Tumour cell killing by tumour necrosis factor: inhibition by anaerobic conditions, free-radical scavengers and inhibitors of arachidonate metabolism. Immunology **62**:153–155.
48. **Mestan, J., W. Digel, S. Mittnacht, H. Hillen, D. Blohm, A. Moller, H. Jacobsen, and H. Kirchner.** 1986. Antiviral effects of recombinant tumour necrosis factor *in vitro*. Nature (London) **323**:816–819.
49. **Mortensen, R. F., J. Shapiro, B.-F. Lin, S. Douches, and R. Neta.** 1988. Interaction of recombinant IL-1 and recombinant tumor necrosis factor in the induction of mouse acute phase proteins. J. Immunol. **140**:2260–2266.
50. **Munker, R., J. Gasson, M. Ogawa, and H. P. Koeffler.** 1986. Recombinant human TNF induces production of granulocyte-monocyte colony-stimulating factor. Nature (London) **323**:79–82.
51. **Ostensen, M. E., D. L. Thiele, and P. E. Lipsky.** 1987. Tumor necrosis factor-α enhances cytolytic activity of human natural killer cells. J. Immunol. **138**:4185–4191.
52. **Paabo, S., B. M. Bhat, W. S. M. Wold, and P. A. Peterson.** 1987. A short sequence in the COOH-terminus makes an adenovirus membrane glycoprotein a resident of the endoplasmic reticulum. Cell **50**:311–317.
53. **Palombella, V. J., D. J. Yamashiro, F. R. Maxfield, S. J. Decker, and J. Vilcek.** 1987. Tumor necrosis factor increases the number of epidermal growth factor receptors on human fibroblasts. J. Biol. Chem. **262**:1950–1954.
54. **Pavlakis, G. N., and D. H. Hamer.** 1983. Regulation of a metallothionein-growth hormone hybrid gene in bovine papilloma virus. Proc. Natl. Acad. Sci. USA **80**:397–401.
55. **Peetre, C., H. Thysell, A. Grubb, and I. Olsson.** 1988. A tumor necrosis factor binding protein is present in human biological fluids. Eur. J. Haematol. **41**:414–419.
56. **Pessara, U., and N. Koch.** 1990. Tumor necrosis factor α regulates expression of the major histocompatibility complex class II-associated invariant chain by binding of an NF-κB-like factor to a promoter element. Mol. Cell. Biol. **10**:4146–4154.
57. **Pfizenmaier, K., P. Scheurich, C. Schluter, and M. Kronke.** 1987. Tumor necrosis factor enhances HLA-A,B,C and HLA-DR gene expression in human tumor cells. J. Immunol. **138**:975–980.
58. **Pratt, L. H., W. McCurdy, Y. Shimazaki, and M.-M. Cordonnier.** 1986. Immunodetection of phytochrome: immunochemistry, immunoblotting, and immunoquantitation. Mod. Methods Plant Anal. **4**:50–74.
59. **Priore, R. L., and H. E. Rosenthal.** 1976. A statistical method for the estimation of binding parameters in a complex system. Anal. Biochem. **70**:231–240.
59a. **Ranheim, T. S., and W. S. M. Wold.** Unpublished data.
60. **Rawle, F. C., A. E. Tollefson, W. S. M. Wold, and L. R. Gooding.** 1989. Anti-adenovirus cytotoxic T lymphocytes. Inhibition of lysis by E3 gp19K but not E3 14.7K. J. Immunol. **143**:2031–2037.
61. **Ruggiero, V., K. Latham, and C. Baglioni.** 1987. Cytostatic and cytotoxic activity of tumor necrosis factor on human cancer cells. J. Immunol. **138**:2711–2717.
62. **Scanlon, M., S. M. Laster, J. G. Wood, and L. R. Gooding.** 1989. Cytolysis by tumor necrosis factor is preceded by a rapid and specific dissolution of microfilaments. Proc. Natl. Acad. Sci. USA **86**:182–186.
63. **Scheurich, P., B. Thoma, U. Ucer, and K. Pfizenmaier.** 1987. Immunoregulatory activity of recombinant human tumor necrosis factor (TNF)-α: induction of TNF receptors on human T cells and TNF-α-mediated enhancement of T cell responses. J. Immunol. **138**:1786–1790.
64. **Schutze, S., S. Nottrott, K. Pfizenmaier, and M. Kronke.** 1990. Tumor necrosis factor signal transduction. Cell-type-specific activation and translocation of protein kinase C. J. Immunol. **144**:2604–2608.
65. **Schutze, S., P. Scheurich, K. Pfizenmaier, and M. Kronke.** 1989. Tumor necrosis factor signal transduction. Tissue-specific serine phosphorylation of a 26-kDa cytosolic protein. J. Biol. Chem. **264**:3562–3567.
66. **Seckinger, P., S. Isaaz, and J.-M. Dayer.** 1988. A human inhibitor of tumor necrosis factor α. J. Exp. Med. **167**:1511–1516.
67. **Shalaby, M. R., B. B. Aggarwal, E. Rinderknecht, L. P. Svedersky, B. S. Finkle, and M. A. Palladino, Jr.** 1985. Activation of human polymorphonuclear neutrophil functions by interferon-gamma and tumor necrosis factors. J. Immunol. **135**:2069–2073.
68. **Shau, H.** 1988. Characteristics and mechanism of neutrophil-mediated cytostasis induced by tumor necrosis factor. J. Immunol. **141**:234–240.
69. **Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson, and D. C. Klenk.** 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**:76–85.
70. **Smith, R. A., and C. Baglioni.** 1989. Multimeric structure of the tumor necrosis factor receptor of HeLa cells. J. Biol. Chem. **264**:14646–14652.
71. **Strieter, R. M., S. L. Kunkel, H. J. Showell, D. G. Remick, S. H. Phan, P. A. Ward, and R. M. Marks.** 1989. Endothelial cell gene expression of a neutrophil chemotactic factor by TNF-α, LPS, and IL-1β. Science **243**:1467–1469.
72. **Sugarman, B. J., B. B. Aggarwal, P. E. Hass, I. S. Figari, M. A. Palladino, Jr., and H. M. Shepard.** 1985. Recombinant human tumor necrosis factor-α: effects on proliferation of normal and

transformed cells *in vitro*. Science **230**:943–945.

73. **Tollefson, A. E., and W. S. M. Wold.** 1988. Identification and gene mapping of a 14,700-molecular-weight protein encoded by region E3 of group C adenoviruses. J. Virol. **62**:33–39.
74. **Torti, F. M., B. Dieckmann, B. Beutler, A. Cerami, and G. M. Ringold.** 1985. A macrophage factor inhibits adipocyte gene expression: an *in vitro* model of cachexia. Science **229**:867–869.
75. **Tracey, K. J., Y. Fong, D. G. Hesse, K. R. Manogue, A. T. Lee, G. C. Kuo, S. F. Lowry, and A. Cerami.** 1987. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature (London) **330**:662–664.
76. **Tsujimoto, M., Y. K. Yip, and J. Vilcek.** 1985. Tumor necrosis factor: specific binding and internalization in sensitive and resistant cells. Proc. Natl. Acad. Sci. USA **82**:7626–7630.
77. **Vaessen, R. T. M. J., A. Houweling, P. Kourilsky, and A. J. van der Eb.** 1986. Adenovirus E1A-mediated regulation of class I MHC expression. EMBO J. **5**:335–341.
78. **Vilcek, J., V. J. Palombella, D. Henriksen-DeStefano, C. Swenson, R. Feinman, M. Hirai, and M. Tsujimoto.** 1986. Fibroblast growth enhancing activity of tumor necrosis factor and its relationship to other polypeptide growth factors. J. Exp. Med. **163**:632–643.
79. **Watanabe, N., H. Kuriyama, H. Sone, H. Neda, N. Yamauchi, M. Maeda, and Y. Niitsu.** 1988. Continuous internalization of tumor necrosis factor receptors in a human myosarcoma cell line. J. Biol. Chem. **263**:10262–10266.
80. **Whyte, P., N. M. Williamson, and E. Harlow.** 1989. Cellular targets for transformation by the adenovirus E1A proteins. Cell **56**:67–75.
81. **Wold, W. S. M., S. L. Deutscher, N. Takemori, B. M. Bhat, and S. C. Magie.** 1986. Evidence that AGUAUAUGA and CCAA GAUGA initiate translation in the same mRNA in region E3 of adenovirus. Virology **148**:168–180.
82. **Wong, G. H. W., J. H. Elwell, L. W. Oberley, and D. V. Goeddel.** 1989. Manganous superoxide dismutase is essential for cellular resistance to cytotoxicity of tumor necrosis factor. Cell **58**:923–931.
83. **Wong, G. H. W., and D. V. Goeddel.** 1986. Tumour necrosis factors α and β inhibit virus replication and synergize with interferons. Nature (London) **323**:819–822.
84. **Wong, G. H. W., and D. V. Goeddel.** 1988. Induction of manganous superoxide dismutase by tumor necrosis factor: possible protective mechanism. Science **242**:941–944.
85. **Wong, G. H. W., J. F. Krowka, D. P. Stites, and D. V. Goeddel.** 1988. In vitro anti-human immunodeficiency virus activities of tumor necrosis factor-α and interferon-gamma. J. Immunol. **140**:120–124.