The Adenovirus E3-14.7K Protein and the E3-10.4K/14.5K Complex of Proteins, Which Independently Inhibit Tumor Necrosis Factor (TNF)-Induced Apoptosis, Also Independently Inhibit TNF-Induced Release of Arachidonic Acid

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Tumor necrosis factor (TNF) is an inflammatory cytokine that inhibits the replication of many viruses in cultured cells. We have reported that adenovirus (Ad) infection of TNF-resistant mouse cells renders them susceptible to lysis by TNF and that two sets of proteins encoded by the E3 transcription unit block TNF cytotoxicity. The E3 protein sets are named E3-14.7K (14,700 kDa) and E3-10.4K/14.5K (a complex of two proteins of 10,400 and 14,500 kDa). TNF activation of the 85-kDa cytosolic phospholipase A₂ (cPLA₂) is thought to be essential for TNF cytotoxicity (i.e., TNF-induced apoptosis). Here we provide evidence that cPLA₂ is important in the response of Ad-infected cells to TNF and that the mechanism by which E3-14.7K and E3-10.4K/14.5K inhibit TNF cytotoxicity is by inhibiting TNF activation of cPLA₂. cPLA₂ cleaves arachidonic acid (AA) specifically from membrane phospholipids; therefore, cPLA₂ activity was measured by the release of ³H-AA from cells prelabeled with ³H-AA. Uninfected cells or cells infected with wild-type Ad were not lysed and did not release ³H-AA in response to TNF. In contrast, TNF treatment induced cytotoxicity and ³H-AA release in uninfected cells sensitized to TNF by treatment with cycloheximide and also in infected cells sensitized to TNF by expression of E1A. In C127 cells, in which either E3-14.7K or E3-10.4K/14.5K inhibits TNF cytotoxicity, either set of proteins inhibited TNF-induced release of ³H-AA. In C3HA cells, in which E3-14.7K but not E3-10.4K/14.5K prevents TNF cytotoxicity, E3-14.7K but not E3-10.4K/14.5K prevented TNF-induced release of ³H-AA. When five virus mutants with lesions in E3-14.7K were examined, there was a perfect correlation between a mutant's ability to inhibit both TNF-induced cytotoxicity and release of ³H-AA. E3-14.7K expressed in two stably transfected C127 cell lines prevented both TNF-cycloheximide-induced cytotoxicity and release of ³H-AA. The E3 proteins also prevented TNF-induced cytotoxicity and release of ³H-AA in mouse L929 cells, which are spontaneously sensitive to TNF. TNF cytotoxicity was blocked by dexamethasone, an inhibitor of PLA₂ activity, and by nordihydroquaiaric acid, which inhibits the metabolism of AA to the leukotrienes. Indomethacin, which blocks the formation of prostaglandins from AA, did not inhibit TNF cytotoxicity. The leukotrienes and prostaglandins are amplifiers of the inflammatory response. We propose that E3-14.7K and E3-10.4K/14.5K function independently in Ad infection to inhibit both cytotoxicity and inflammation induced by TNF.

Infections or other types of tissue injury lead to an inflammatory response, a complex process that culminates in the elimination of infection and tissue repair (reviewed in reference 2). Tumor necrosis factor (TNF) and interleukin-1 are the major cytokines that initiate inflammation (reviewed in reference 73). TNF synthesis is induced in mice by different RNA and DNA viruses, including human adenovirus (Ad) (17), and TNF has been proposed to have antiviral effects *in vivo* (23, 84). TNF inhibits the replication in cultured cells of RNA and DNA viruses, including Ad (89), and it lyses cells infected with different viruses (reviewed in references 18 and 19).

TNF must be important in Ad infection because Ad encodes several proteins that affect the cellular response to TNF (re-

viewed in references 18, 23, and 83 to 87). Cultured mouse cells infected with Ad are not normally killed by TNF, but they are killed when infected with certain virus mutants that lack genes in the E3 transcription unit (20). This finding indicates that Ad sensitizes cells to TNF, and E3 encodes proteins that inhibit cytotoxicity by TNF. Studies with Ad mutants indicate that the E1A proteins sensitize infected cells to TNF cytotoxicity (13, 14, 64). Expression of E1A in stably transfected cells also sensitizes them to TNF (1, 8, 13, 35, 64, 74, 78). Regarding the E3 proteins that inhibit TNF cytotoxicity, there are two sets of proteins that have this function, depending on the cell line. The E3 14,700-kDa (E3-14.7K) protein inhibited TNF cytotoxicity in 13 of 15 mouse cell lines analyzed (20, 21). The E3 10,400-kDa and 14,500-kDa proteins, which function as a heterodimer named E3-10.4K/14.5K (71), inhibited TNF cytotoxicity in 11 of 15 mouse cell lines examined (21). In human (but not mouse) cells, the Ad E1B-19K protein inhibits TNF cytotoxicity of infected (19) or transfected (82) cells. In the absence of E1B-19K, E3 proteins inhibit TNF cytotoxicity of human cells, but it is

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not known if this is due to E3-14.7K, E3-10.4K/14.5K, or both sets of proteins (19).

What is the mechanism by which these three sets of proteins independently inhibit TNF cytotoxicity? This is a difficult question because the mechanism of TNF cytotoxicity is poorly understood (reviewed in reference 29). TNF kills most cell types by apoptosis rather than necrosis (45), but it is not known if TNF-induced apoptosis occurs by the same final mechanism as apoptosis induced by other means (reviewed in reference 49). Some tumor cell lines are spontaneously sensitive to TNF cytotoxicity, but most cell lines are resistant. Most of the latter cells can be sensitized to TNF by inhibitors of transcription or translation. These cells may possess a preexisting latent mechanism for cytotoxicity that becomes activated by TNF in the absence of protein synthesis; this process could be analogous to the induction of apoptosis in the absence of protein synthesis or even in the absence of nuclei (49). Another possibility is that TNF induces proteins that inhibit TNF cytotoxicity (36), such as manganous superoxide dismutase (88), plasminogen activator inhibitor type 2 (44), and the transcription factor A20 (55); induction of these proteins would be inhibited by cycloheximide (CHX), and consequently the cell would undergo cytotoxicity. Regardless of the scenario, E3-14.7K, E3-10.4K/14.5K, and E1B-19K inhibit TNF cytotoxicity when the cells are sensitized to TNF by CHX (19–22, 33). E3-14.7K also inhibits cytotoxicity when cells are sensitized to TNF by cytochalasin E (22).

There is considerable evidence that TNF cytotoxicity requires the activation of phospholipase A₂ (PLA₂). PLA₂ activity is typically measured by labeling cells with ³H-labeled arachidonic acid (AA), which is incorporated nearly exclusively into the sn2 position of membrane phospholipids (37), and then observing the release from cells of ³H-AA in response to TNF. Most studies have been with cells that are spontaneously sensitive to TNF cytotoxicity, such as the mouse L929 and WEHI-164 and human U937 and MCF-7 cell lines, and the involvement of PLA₂ was further indicated by the use of inhibitory drugs (34, 37, 50, 53, 59, 68). Other studies have used TNF-resistant cells that must be sensitized to TNF by, e.g., CHX. These cell types do not lyse or release AA in response to TNF, but they do so when treated with TNF plus CHX; again, inhibitors of PLA₂ prevent TNF-induced cytotoxicity and release of AA (32, 52, 59, 80, 91). Further indication for the role of PLA₂ in TNF cytotoxicity comes from the isolation of TNF-resistant cell lines that are defective in TNF-induced release of AA (28, 53, 61, 62, 68). There are different species of PLA₂: several species of ca. 14 kDa that are secreted, and an 85-kDa cytosolic PLA₂ (cPLA₂) (reviewed in reference 12). There is strong evidence that TNF must activate cPLA₂ in order for cytotoxicity and AA release to occur. cPLA₂ is normally in the cytosol in an inactive form. It can be activated via phosphorylation by mitogen-activated protein kinase (48, 54), and in the presence of calcium, cPLA₂ translocates to membranes to cleave AA specifically from the sn2 position of membrane phospholipids (10, 15). cPLA₂ is activated by many agonists, including TNF (30), interleukin-1, growth factors, and phorbol esters (10, 46, 47). Voelkel-Johnson et al. (80) reported a near-perfect correlation between the activity of cPLA₂ per cell and susceptibility to cytotoxicity by TNF in several melanoma and mouse cell lines that were sensitized to TNF by using CHX. Also, cytotoxicity by TNF plus CHX was increased by overexpression of cPLA₂ and decreased by antisense oligonucleotides to cPLA₂ (80). Hayakawa et al. (28), working with L929 cells, isolated TNF-resistant cells that lack cPLA₂; transfection of the cPLA₂ cDNA into these cells partially restored susceptibility to TNF.

In this report, we addressed whether cPLA₂ is involved in

TNF-induced cytotoxicity in Ad-infected mouse cells that are normally resistant to TNF and also in L929 cells that are spontaneously sensitive to TNF. We found that Ad sensitizes TNF-resistant cells to TNF-induced release of AA, just as it does to TNF-induced cytotoxicity, and that the E3-14.7K and E3-10.4K/14.5K proteins inhibit TNF-induced release of AA. The E3 proteins also block TNF-induced cytotoxicity and AA release in L929 cells. This finding suggests that the mechanism by which E3-14.7K and E3-10.4K/14.5K prevent TNF cytotoxicity is by preventing TNF-induced synthesis of AA, very likely by inhibiting TNF-activation of cPLA₂. AA can be metabolized to prostaglandins and leukotrienes; these eicosanoids mediate the inflammatory response. Thus, the function of E3-14.7K and E3-10.4K/14.5K in Ad biology may be not only to inhibit TNF cytotoxicity but also to prevent a TNF-induced inflammatory response.

MATERIALS AND METHODS

Cells and viruses. Mouse C127, C3HA, and L929 cells were grown in Dulbecco's minimal essential medium (DME) containing 10% fetal bovine serum (FBS).

Virus stocks were prepared in suspension cultures of human KB cells and titrated on human A549 cells (24). *rec700* is an Ad type 5 (Ad5)-Ad2-Ad5 recombinant containing the Ad5 *EcoRI*-A (map positions 0 to 76), Ad2 *EcoRI*-D (map positions 76 to 83), and Ad5 *EcoRI*-C (map positions 83 to 100) fragments. *rec700* is the wild-type parent for *dl748*, *dl753*, *dl758*, and *dl763* (5, 6). With respect to E3 proteins, *dl748* expresses only the E3-14.5K protein, *dl753* lacks only E3-10.4K, *dl758* lacks only E3-14.7K, and *dl763* lacks E3-14.5K and E3-14.7K (20, 69, 70).

dl7001 lacks all E3 genes, and *dl7000* lacks all E3 genes except E3-14.7K (60). The following mutants are in a *dl7000* background: *pm7014* (Cys-104 in E3-14.7K converted to Ser), *pm7015* (Cys-112 to Ser), *dl7020* (Δ 2-7, i.e., residues 2 to 7 in E3-14.7K deleted), *dl7022* (Δ 18-33), and *dl7032* (Δ 122-128) (60).

Isolation of C127 cell lines expressing the E3-14.7K protein. The gene for E3-14.7K was cloned behind the human cytomegalovirus promoter to generate pCMV-14.7K. pCMV-14.7K (1 μ g) was mixed with 1 μ g of p525B (77), 0.1 μ g of pSV2_{neo}, and 8 μ g of salmon sperm DNA, coprecipitated with calcium phosphate, and transfected into C127 cells. At 48 h posttransfection, the cells were split into two 75-cm² flasks, and transfected cells were selected by growth in medium containing 1 mg of G418 (GibcoBRL, Gaithersburg, Md.) per ml. The cells were maintained in G418-containing medium. Single-cell clone 12-14 was isolated by limited dilution of cells into 96-well dishes. Similarly, pCMV-14.7K, p59 t1 2878 (90), and pSV2_{neo} were used to isolate G418-resistant cells. Single-cell clone 20-2E was isolated by treating these cells with 50 U of TNF plus 2.5 μ g CHX per ml for 22 h. All 20-2E and 12-14 cells expressed high levels of E3-14.7K, as indicated by immunofluorescence.

AA release experiments. C127, C3HA, or L929 cells were split 20 to 22 h before the experiment. Cells were trypsinized; 8×10^5 cells were plated onto 60-mm-diameter plates and after 3 h were infected in 500 μ l of DME-0.5% FBS with 600 to 800 PFU of the indicated virus per cell. After 1 h, 4.5 ml of DME-10% FBS was added. At 6 h postinfection (p.i.), cells were washed with DME-10% FBS and labeled overnight in 2 ml of complete medium containing 7 μ Ci of [5, 6, 8, 11, 12, 14, 15-³H]AA (180 to 240 Ci/mmol; NEN Dupont Research Products, Boston, Mass.) per ml. At 20 h p.i., cells were rinsed twice with 5 ml of Hanks' balanced salt solution, trypsinized, and seeded into 96-well plates (10^4 cells per well). After 3 h, cells were rinsed with 150 μ l of Hanks' balanced salt solution and treated with 100 μ l of DME-10% FBS containing mouse TNF (Boehringer Mannheim Corp., Indianapolis, Ind.) (TNF concentrations are indicated in legends) and 20 μ g of CHX (Sigma Chemical Company, St. Louis, Mo.) per ml. At times indicated, plates were centrifuged (Beckman GC-6R centrifuge, 5 min, 2,000 rpm, room temperature), and 75 μ l was transferred into vials for counting. Each time point was determined in triplicate.

To determine the total incorporation of ³H-AA, cells were solubilized in 50% DME-H₂O containing 20% sodium dodecyl sulfate (SDS) and counted. Specific release was computed as follows: (release [treated] – release [spontaneous]) / (total incorporation – release [spontaneous]). The total incorporation of ³H-AA ranged from 10,000 to 30,000 cpm.

To characterize the ³H-labeled eicosanoids, 500 μ l of culture supernatant (DME-10% FBS) or sonicated lysed cell extract was added to 4.5 ml of cold methanol and mixed well. After 1 h at –20°C, samples were centrifuged at 1,800 $\times$ g at –15°C for 20 min. The protein pellet was resuspended in 1 ml of cold methanol and recentrifuged. The methanolic supernatants were combined and centrifuged under reduced atmosphere at room temperature until dry. The dried samples were dissolved in 400 μ l of the high-pressure liquid chromatography (HPLC) mobile phase. Metabolites of ³H-AA were separated by reverse-phase HPLC on a Luceosil 5 μ m C₁₈ column, using a linear solvent gradient from CH₃CN-H₂O-CH₃COOH (49.95:49.95:0.1) to CH₃CN-CH₃COOH

(99.9:0.1) over 40 min at 1 ml/min. Radioactivity was measured by on-line liquid scintillation detection, using a Radiomatic Flo-One β -detector (Packard, Meriden, Conn.). Elution times of sample peaks were compared with those of authentic eicosanoid standards.

LDH release. Lactate dehydrogenase (LDH) release is an established method to measure cell viability (11, 40). Cells were infected, seeded into 96-well plates, and treated with mouse TNF or TNF plus CHX as described above for ^3H -AA release. Twenty-microliter samples of culture supernatant were removed and assayed for LDH release by the Cytotox 96 assay (Promega Biotech, Madison, Wis.). Samples were assayed in triplicate on an EL340 Microplate reader (Bio-Tec Instruments, Inc.) at 490 nm.

MTT assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay for mitochondrial activity, an indicator of cell viability (27, 51), was carried out on cells in 96-well plates at 10 h after treatment with mouse TNF and 20 μg of CHX per ml. Twenty-five microliters of MTT (5 mg/ml; Sigma) was added, the cells were incubated for 2 h in a CO_2 incubator at 37°C , 100 μl of lysis buffer (20% SDS in 50% dimethylformamide- H_2O [pH 4.7]) was added, and cells were incubated overnight. The A_{570} was measured in an enzyme-linked immunosorbent assay reader. The assays were performed in triplicate.

Drug treatments. If not indicated, drugs were added 3 to 4 h before TNF treatment (after plating cells into the 96-well plates) at the concentration indicated. Drugs were present during TNF treatment.

RESULTS

The ^3H counts released from cells undergoing TNF cytotoxicity are ^3H -AA. This report addresses the relationship between TNF cytotoxicity and TNF activation of PLA_2 , presumably cPLA_2 , in mouse C3HA, C127, and L929 cells. We employed a commonly used in vivo assay for PLA_2 , namely, prelabeling cells with ^3H -AA and then measuring the release from cells of ^3H -AA when the cells are undergoing cytotoxicity in response to TNF. The cytotoxicity and ^3H -AA release data will be discussed below; here we examine the ^3H -AA counts released. Hollenbach et al. (32) showed that nearly all of the ^3H counts released from C3HA cells lysed by TNF-CHX are in fact ^3H -AA. This is also the case for C127 cells lysed by TNF-CHX (Fig. 1A), *dl763* (E3-14.5K⁻, E3-14.7K⁻)-infected C127 cells lysed by TNF (Fig. 1C), and L929 cells lysed by TNF alone (data not shown). Other workers have also found that ^3H -AA accounts for most of the ^3H counts released from TNF-treated L929 cells (68). Almost all of the intracellular unincorporated ^3H counts were also ^3H -AA (Fig. 1B and D). Since cPLA_2 cleaves only AA from lipids, since only ^3H -AA was released, and given the other strong data implicating cPLA_2 in TNF-induced release of AA (see the introduction), these results strongly suggest that the ^3H -AA release observed in our experiments is caused by cPLA_2 .

TNF-induced cytotoxicity correlates with TNF-induced release of AA in Ad-infected cells that are sensitized to TNF by expression of E1A or inhibition of protein synthesis. As discussed, there is evidence that TNF-induced release of AA correlates with TNF cytotoxicity and that it may be necessary for TNF cytotoxicity. For example, when mock-infected C3HA cells labeled with ^3H -AA were untreated or treated with TNF, they neither lysed nor released ^3H -AA (Fig. 2A and B). However, when treated with TNF-CHX, mock-infected cells lysed efficiently and up to 17% of the total ^3H -AA incorporated was released (Fig. 2C and D). This finding confirms the results of Zilli et al. (91). Similar results were observed with C127 cells (e.g., Fig. 4 to 8).

C3HA cells are not lysed by TNF when infected with wild-type Ads, but they are lysed by TNF when infected with mutants that lack E3-14.7K (20–22). Accordingly, we asked whether there is a correlation between TNF cytotoxicity and TNF-induced release of AA. With *rec700* (wild type), there was little killing of cells after treatment for 10 h with a high concentration of TNF (200 U/ml), and there was little specific release of ^3H -AA (Fig. 2A and B). When *rec700*-infected cells were treated with TNF-CHX (which is much more cytotoxic than

FIG. 1. Analysis of ^3H counts released in C127 cells undergoing cytotoxicity in response to TNF. (A and B) Extracellular and intracellular ^3H counts, respectively, from cells treated with 200 U of TNF plus 25 μg of CHX per ml; (C and D) extracellular and intracellular ^3H counts released from *dl763*-infected cells treated with TNF (200 U). The samples were taken 7.5 h after addition of TNF, a period when they are undergoing lysis. The ^3H counts were analyzed by HPLC under conditions standardized to detect AA and related lipids.

TNF alone), most of the cells remained viable and there was negligible specific release of ^3H -AA (Fig. 2C and D). With *dl763* (E3-10.4K⁺, E3-14.5K⁻, E3-14.7K⁻), efficient cytotoxicity and ^3H -AA release occurred in cells treated with TNF alone (Fig. 2A and B) or TNF-CHX (Fig. 2C and D). The *dl763*-infected cells treated with TNF alone were lysed because they were sensitized to TNF by expression of E1A (13, 14, 64) and because the mutant lacks E3-14.7K, which inhibits TNF cytotoxicity. Thus, in Ad-infected C3HA cells, there is indeed a correlation between TNF cytotoxicity and release of ^3H -AA. Ad infection and/or treatment with CHX sensitize cells to TNF-induced release of ^3H -AA. Presumably the E1A proteins sensitize the cells to TNF-induced release of ^3H -AA just as they do to TNF cytotoxicity (13, 14, 64), and the E3-14.7K protein inhibits TNF-induced release of ^3H -AA just as it inhibits TNF cytotoxicity (20–22).

An important issue is whether TNF-induced release of AA is a cause or an effect of TNF cytotoxicity. If it is a cause, then release of ^3H -AA should precede cytotoxicity. This is clearly the case in TNF-treated C127 cells infected with *dl7001* (E3⁻) (Fig. 3). These results are in accord with studies in cell lines that are spontaneously sensitive to TNF (37, 53, 59, 68) or that were sensitized to TNF by CHX (32, 91). Also consistent with ^3H -AA release (i.e., cPLA_2 activation) being a cause and not an effect of TNF cytotoxicity, only 3 to 4% of ^3H -AA was released by freezing and thawing or sonication of the cells (data not shown), whereas up to 40% of the incorporated ^3H -AA was released specifically in response to TNF (e.g., Fig. 6 to 8 and 10). That is, the ^3H -AA released is not simply the result of cell lysis; rather, it must be due to cPLA_2 activity. In similar studies, <2 to 5% of unincorporated intracellular ^3H -AA was observed (34, 37, 52, 57).

FIG. 2. TNF-induced cytolysis correlates with TNF-induced release of $^3\text{H-AA}$ in C3HA cells. Cells were mock infected or infected with *rec700* (wild type) or *dl763* (E3-10.4K^+ , E3-14.5K^- , E3-14.7K^-). Half of the dishes were labeled with $^3\text{H-AA}$ ($1 \mu\text{Ci/ml}$). At 24 h p.i., cells were treated with mouse TNF (600 U/ml) or TNF plus CHX ($25 \mu\text{g/ml}$), and $^3\text{H-AA}$ release was determined. The remaining dishes were treated with TNF or TNF-CHX at 24 h p.i. as indicated; cytolysis was determined 16 h later by LDH release. (A) LDH release, cells treated with TNF; (B) $^3\text{H-AA}$ release, cells treated with TNF; (C) LDH release, cells treated with TNF-CHX; (D) $^3\text{H-AA}$ release, cells treated with TNF-CHX.

If TNF activation of PLA_2 is a cause of TNF cytolysis, then inhibitors of PLA_2 should inhibit TNF cytolysis. Dexamethasone, an inhibitor of PLA_2 activity, inhibited both $^3\text{H-AA}$ release and cytolysis induced by TNF-CHX in mock-infected C127 cells (Fig. 4A) and in cells infected with *dl7001* and treated with TNF (Fig. 4B) or TNF-CHX (Fig. 4C). Nordihydroguaiaretic acid (NDGA) inhibits PLA_2 at concentrations of $>5 \mu\text{M}$ (4, 56); $10 \mu\text{M}$ NDGA strongly inhibited $^3\text{H-AA}$ release and cytolysis in response to TNF or TNF-CHX (Fig. 5).

AA can be metabolized via the lipoxygenase or cyclooxygenase pathway. NDGA at $<5 \mu\text{M}$ inhibits the lipoxygenases (3).

FIG. 3. Kinetics of TNF-induced $^3\text{H-AA}$ release and TNF-induced cytolysis in *dl7001*-infected C127 cells. Cells were infected, labeled with $1 \mu\text{Ci}$ of $^3\text{H-AA}$ per ml, and treated with 250 U of mouse TNF per ml, and $^3\text{H-AA}$ release (open squares) and LDH release (closed squares) were measured at the times indicated.

FIG. 4. Inhibition of TNF-induced $^3\text{H-AA}$ release and cytotoxicity by dexamethasone (Dex). C127 cells were mock infected (A), infected with *dl7001* (B), or infected with *dl7001* plus CHX (C) and labeled with $^3\text{H-AA}$ ($1 \mu\text{Ci/ml}$). Cells were untreated or treated with $1 \mu\text{M}$ dexamethasone for 4 or 18 h prior to treatment with 250 U of mouse TNF per ml (B) or 250 U of TNF plus $20 \mu\text{g}$ of CHX per ml (A and C). $^3\text{H-AA}$ release and LDH release were measured 10 h posttreatment.

As shown in Fig. 5, 3.3 and $10 \mu\text{M}$ NDGA inhibited both release of $^3\text{H-AA}$ and cytolysis induced by TNF. Importantly, $20 \mu\text{M}$ indomethacin, an inhibitor of the cyclooxygenase pathway, did not inhibit either $^3\text{H-AA}$ release or cytolysis.

Altogether, these results suggest that TNF activation of cPLA_2 is necessary for TNF-induced cytolysis in C127 and C3HA cells when the cells are sensitized to TNF either by treatment with CHX or by expression of E1A and that the lipoxygenase pathway rather than the cyclooxygenase pathway is involved in TNF cytolysis.

The ability of an Ad mutant to inhibit TNF cytolysis correlates with its ability to inhibit TNF-induced release of AA in cells that must be sensitized to TNF and also in L929 cells that are spontaneously sensitive to TNF. In C127 cells infected with Ad E3 mutants, either E3-14.7K or E3-10.4K/14.5K can independently inhibit TNF cytolysis (21). This is also true for TNF-induced release of AA. As shown in Fig. 6, large amounts of $^3\text{H-AA}$ were released by TNF-CHX in mock-infected C127

FIG. 5. Inhibition of TNF-induced ^3H -AA release and cytolysis by inhibitors of PLA₂ and the lipoxygenase pathway. C127 cells were mock infected (A and B), infected with *dl7001* (C and D), or infected with *dl7001* plus CHX (E and F). After labeling with ^3H -AA (1 $\mu\text{Ci}/\text{ml}$), the cells were untreated or treated with 1 μM NDGA, 3.3 μM NDGA, 10 μM NDGA, or 20 μM indomethacin. Three hours later, 250 U of mouse TNF per ml (C and D) or 250 U of TNF plus 20 μg of CHX per ml (A, B, E, and F) were added. ^3H -AA release (A, C, and E) and LDH release (B, D, and F) were determined 10 h following treatment.

cells and in cells infected with *dl7001* (E3^-), *dl763* (expresses all E3 proteins except E3-14.5K and E3-14.7K), or *dl748* (the only E3 protein expressed in abundance is E3-14.5K). Much less ^3H -AA was released by TNF-CHX in cells infected with *rec700* (wild type), *dl758* (expresses all E3 proteins except E3-14.7K), or *dl7000* (the only E3 protein expressed is E3-14.7K). Thus, TNF-CHX-induced release of ^3H -AA was inhibited by E3-14.7K alone (*dl7000*) or in the absence of E3-14.7K by the E3-10.4K/14.5K complex (shown by the result with

dl763). When cells were coinfecting with *dl763* (which provides E3-10.4K) and *dl748* (which provides E3-14.5K), complementation was observed (Fig. 6), confirming that the E3-10.4K/14.5K complex inhibits TNF-CHX-induced release of ^3H -AA in the absence of E3-14.7K. These ^3H -AA release results parallel our TNF cytolysis results with these same mutants (21).

In C3HA cells, the E3-14.7K protein and no other Ad protein is required to inhibit TNF cytolysis (20–22). This is also true with respect to TNF-induced release of AA. Mock-infected C3HA cells released ^3H -AA in response to TNF-CHX but not to TNF alone, and *rec700*-infected cells released only minimal ^3H -AA in response to TNF-CHX (Fig. 7A). However, in cells infected with *dl758* (expresses all E3 proteins except E3-14.7K), ^3H -AA was released in response to TNF or TNF-CHX. In the same experiment with C127 cells, neither TNF nor TNF-CHX induced release of ^3H -AA in cells infected with *dl758* or *rec700* (Fig. 7B). Thus, as is the case with cytolysis, E3-10.4K/14.5K inhibits TNF-induced release of ^3H -AA in C127 cells but not in C3HA cells, and E3-14.7K inhibits TNF-induced release of ^3H -AA in both C3HA and C177 cells.

We have described a series of virus mutants with in-frame deletions and Cys-to-Ser missense mutations in the E3-14.7K protein, built into a *dl7000* background (E3-14.7K is the only E3 protein expressed), and characterized for their ability to inhibit TNF cytolysis (60). When a subset of these mutants was examined for inhibition of ^3H -AA release in response to TNF-CHX, some mutants were able to inhibit whereas others were

FIG. 6. The E3-14.7K protein and the E3-10.4K/14.5K complex of proteins independently inhibit TNF-induced release of ^3H -AA in C127 cells. Cells were infected with the indicated virus and labeled with ^3H -AA (1 $\mu\text{Ci}/\text{ml}$). ^3H -AA release was determined following treatment with mouse TNF (500 U/ml) plus CHX (20 $\mu\text{g}/\text{ml}$).

FIG. 7. The E3-10.4K/14.5K complex inhibits TNF-induced ^3H -AA release in C127 cells but not in C3HA cells. C3HA (A) or C127 (B) cells were infected with the virus indicated and labeled with ^3H -AA (1 $\mu\text{Ci}/\text{ml}$). ^3H -AA release was measured following treatment with mouse TNF (500 U/ml), CHX (25 $\mu\text{g}/\text{ml}$), or TNF plus CHX. CHX alone did not cause release of ^3H -AA (not shown). Symbols represent mock-infected cells (TNF [open squares]; TNF-CHX [closed squares]), *rec700* (wild-type)-infected cells (TNF [open circles]; TNF-CHX [closed circles]), and *dl758* (E3-10.4K⁺, E3-14.5K⁺, E3-14.7K⁻)-infected cells (TNF [open triangles]; TNF-CHX [closed triangles]).

defective (Fig. 8). These same mutants were wild type or defective for inhibition of TNF cytotoxicity (60).

The C3HA and C127 cells discussed above must be sensitized to TNF. We therefore examined spontaneously sensitive L929 cells to determine if Ad inhibits TNF-induced cytotoxicity and release of AA and if this inhibition maps to the E3 proteins. TNF-treated mock-infected L929 cells lysed (Fig. 9A) and released ^3H -AA (Fig. 9B) in response to TNF. Both TNF-induced cytotoxicity and release of ^3H -AA were inhibited by *rec700* but not by *dl763* (lacks E3-14.5K and E3-14.7K). *rec700*

FIG. 9. Ad E3 proteins inhibit TNF-induced cytotoxicity and release of ^3H -AA in L929 cells. Cells were mock infected or infected with *rec700* or *dl763* (E3-10.4K⁺, E3-14.5K⁻, E3-14.7K⁻). For cytotoxicity (A), cells were transferred to 96-well plates and treated with mouse TNF (0.02 to 200 U/ml) at 8 h p.i., and cytotoxicity was determined by LDH release at 27 h p.i. For ^3H -AA release (B), cells were labeled for 14 h with ^3H -AA, transferred to 96-well plates, and treated with TNF (200 U/ml) and ^3H -AA release was determined at the indicated time points.

infection also inhibited TNF cytotoxicity and TNF-induced release of ^3H -AA in L929 cells treated with TNF-CHX (data not shown).

Altogether, these studies indicate that there is a strict correlation between a mutant's ability to inhibit TNF cytotoxicity and to inhibit TNF-induced release of AA, and this correlation holds for cells that must be sensitized to TNF and for cells that are spontaneously sensitive to TNF.

The E3-14.7K protein stably expressed in transfected C127 cell lines inhibits both TNF cytotoxicity and TNF-induced release of AA. Stably transfected C3HA cells (33) expressing E3-14.7K are resistant to cytotoxicity and AA release induced by TNF-CHX (91). We have isolated two clonal C127 cell lines, 12-14 and 20-2E, stably expressing E3-14.7K. The parental C127 cells were lysed and released ^3H -AA in response to TNF-CHX, whereas the 12-14 and 20-2E lines were not lysed and did not release ^3H -AA (Fig. 10). The MTT assay for mitochondrial activity was used in Fig. 10; similar results were obtained with the LDH assay (data not shown). These cells were resistant to CHX or to TNF alone (data not shown). The 12-14 and 20-2E lines expressed large amounts of E3-14.7K, as indicated by indirect immunofluorescence (data not shown). Thus, 14.7K stably expressed in C3HA or C127 cells in the absence of other Ad proteins inhibits both cytotoxicity and ^3H -AA release induced by TNF-CHX.

FIG. 8. Analysis of E3-14.7K mutants for inhibition of TNF-induced ^3H -AA release. C127 cells were infected with the indicated virus and labeled with ^3H -AA (1 $\mu\text{Ci}/\text{ml}$). The time course of ^3H -AA release was measured after treatment with mouse TNF (250 U/ml) plus CHX (20 $\mu\text{g}/\text{ml}$).

FIG. 10. The E3-14.7K protein inhibits both TNF-induced cytotoxicity and ³H-AA release in stably transfected C127 cells. The results of ³H-AA release (A) and cell viability (B) following treatment with recombinant human TNF (50 U/ml) plus CHX (20 μ g/ml) are shown. The MTT assay was used as an indicator of cell viability. 12-14 and 20-2E are independent high-expression clones of C127 cells stably transfected with a vector expressing E3-14.7K.

DISCUSSION

Although the molecular mechanism of TNF-induced cytotoxicity is not understood, there is much evidence that TNF activation of cPLA₂ is necessary (see the introduction). Our results strongly suggest that this is also true for TNF cytotoxicity of Ad-infected cells. ³H-AA was released from cells by TNF under conditions in which they were undergoing lysis, i.e., mock-infected C3HA or C127 cells treated with TNF-CHX, Ad E3 mutant-infected C3HA or C127 cells treated with TNF alone or TNF-CHX, or mock-infected or E3 mutant-infected L929 cells treated with TNF alone. Whereas L929 cells are spontaneously sensitive to TNF, C3HA and C127 cells must be sensitized to TNF, either by CHX or by Ad infection (probably expression of E1A). Inhibitors of PLA₂ activity, dexamethasone and 10 μ M NDGA, inhibited TNF-induced cytotoxicity and ³H-AA release in mock- and Ad-infected C127 cells. Dexamethasone has been shown to inhibit synthesis of the cPLA₂ mRNA and protein (30).

AA can be metabolized via the lipoygenase pathway to the leukotrienes and hydroperoxyeicosatetraenoic acids and their hydroxy derivatives or via the cyclooxygenase pathway to the prostaglandins and thromboxanes (2). Inhibitors of the lipoygenase pathway, 3.3 and 10 μ M NDGA, inhibited TNF-induced cytotoxicity of mock- or *d17001* (E3⁻)-infected C127 cells. An inhibitor of the cyclooxygenase pathway, 20 μ M indomethacin, did not inhibit TNF cytotoxicity. Other workers have obtained similar results for TNF cytotoxicity of certain other cell types (7, 62, 68). Thus, keeping in mind that NDGA is not entirely specific to the lipoygenase pathway, metabolites formed via this pathway may play a role in TNF cytotoxicity of Ad-infected cells. There is evidence that AA and certain li-

poygenase metabolites act as second messengers (25, 58, 63); perhaps they activate the cytotoxic apparatus in this manner (7). Another possibility is that oxygen radicals produced by leukotriene metabolism induce cytotoxicity (50).

It is unlikely that AA itself or its metabolites are the direct mediators of TNF cytotoxicity, because addition of exogenous AA did not cause lysis of cells treated with CHX or infected with Ad E3 mutants (41). A similar observation was made for L929 cells (68). Also, phorbol esters, calcium ionophore, interleukin-1 β , ATP, thrombin, or growth factors, which activate cPLA₂ to cause AA release, do not induce cell lysis (41, 46–48). Thus, TNF must activate at least two signals for cytotoxicity to occur, one unknown and the other involving cPLA₂.

Without exception, we found a correlation between a given E3 mutant's ability to inhibit both TNF-induced cytotoxicity and release of ³H-AA. This correlation held true for C3HA cells, in which E3-14.7K but not E3-10.4K/14.5K inhibits TNF-induced cytotoxicity and release of ³H-AA, and for C127 cells, in which either E3-14.7K or E3-10.4K/14.5K inhibits TNF effects. We also found that E3-14.7K expressed in transfected C127 cells can inhibit TNF-CHX-induced cytotoxicity and release of AA in the absence of other Ad proteins. Considering that the AA released by most agonists (46–48), including TNF (30), is due to cPLA₂, that Voelkel-Johnson et al. (79) have reported very compelling data indicating that cPLA₂ mediates AA release in C3HA cells, and that only ³H-AA was released from TNF-lysed C3HA (32) and C127 (Fig. 1) cells, it is almost certain that the TNF-induced release of AA in C3HA and C127 cells is due to cPLA₂. The E3 proteins inhibited cytotoxicity and AA release in infected L929 cells. Again, the AA release is almost certainly caused by cPLA₂, which has been shown to be essential for TNF-induced cytotoxicity and AA release in L929 cells (28). Therefore, our results provide strong evidence that these E3 proteins inhibit TNF cytotoxicity by inhibiting TNF activation of cPLA₂, both in cells that must be sensitized to TNF by E1A or CHX and in cells that are spontaneously sensitive to TNF.

Although E1B-19K inhibits TNF cytotoxicity in human cells, it does not do so in mouse cells (19). Clearly, E1B-19K also does not inhibit TNF-induced release of AA in mouse cells, because cells infected with E3 mutants that express E1B-19K release ³H-AA in response to TNF. We do not know if E1B-19K inhibits TNF-induced release of AA in human cells. However, this seems possible because Bcl-2, a functional homolog of E1B-19K (9, 67), inhibits both apoptosis and release of AA in response to TNF (34).

The C3HA and C127 cells must be sensitized to TNF either by E1A or by inhibition of protein synthesis (32, 33, 80, 91), and so there may be an activity that prevents TNF from activating cPLA₂ (Fig. 11). It is important to note that whereas most aspects of TNF signal transduction occur in minutes (reviewed in references 26, 29, and 38), in our experiments only relatively small amounts of ³H-AA release were observed by 20 min (data not shown); ³H-AA release was not observed in substantial quantity until about 2 to 3 h following addition of TNF. Perhaps the agent that blocks TNF activation of cPLA₂ is a labile protein or enzyme, e.g., a protein kinase or phosphatase, that requires 2 to 3 h to turn over or be inactivated in the presence of CHX (Fig. 11). However, this would not be a good explanation for the mechanism by which E1A sensitizes cells to TNF inasmuch as E1A had been expressed in these cells for about 24 h prior to TNF addition. Thus, it is possible that in cells expressing E1A or treated with CHX, TNF itself deactivates a second blocking step, e.g., via activation of a protein kinase or phosphatase, which gradually leads to the unblocking of the TNF-to-cPLA₂ pathway. L929 cells, which are spontaneously sensitive to TNF but release ³H-AA with the

FIG. 11. Proposed role for adenovirus proteins in TNF-induced cytolysis and inflammation. Uninfected C3HA and C127 cells are resistant to TNF activation of cPLA₂, perhaps because a protein or enzyme blocks the TNF signal transduction pathway. When cells are expressing E1A or are treated with CHX, the blocking activity disappears (in 2 to 3 h), allowing TNF to activate cPLA₂ to cleave AA from membrane phospholipids. L929 cells, which contain cPLA₂ yet are spontaneously sensitive to TNF, may lack this blocking activity. AA is metabolized via the lipoxygenase and cyclooxygenase pathways to the leukotrienes and prostaglandins, respectively. TNF activation of cPLA₂, and possibly the formation of leukotrienes, is necessary for TNF cytolysis. The leukotrienes and prostaglandins are amplifiers of the inflammatory response. The E3-14.7K and E3-10.4K/14.5K proteins independently inhibit TNF activation of cPLA₂, thereby inhibiting both TNF cytolysis and a TNF-induced inflammatory response. TNF must activate at least one additional signal for cytolysis, because activation of cPLA₂ by other agonists, or addition of exogenous AA to cells not treated with TNF, does not induce cytolysis. It is not known whether E3-14.7K or E3-10.4K/14.5K inhibits this second putative signal.

same kinetics as sensitized C3HA and C127 cells, may lack the first but not the second blocking step.

As mentioned, Ad only need express E3-14.7K or E3-10.4K/14.5K to be protected from TNF in mouse C127 cells (21). Similarly, in human cells, if the virus expresses E1B-19K, it need not express the E3 proteins (19). Thus, each of the three sets of proteins can function independently to negate the effects of TNF. This is remarkable considering that these are very different proteins. E3-14.7K is a hydrophilic nonmembrane protein localized in the nucleus and cytoplasm (72). E3-10.4K/14.5K exist as integral membrane proteins localized in part on the cell surface (31, 42, 43, 66). E1B-19K localizes predominantly to the nuclear membrane (81). Presumably, E3-14.7K and E3-10.4K/14.5K block different but essential steps in the pathway from the TNF receptor to cPLA₂ (Fig. 11). This may also be true for E1B-19K.

For E3-10.4K/14.5K, the step inhibited could be at the cell surface, perhaps the TNF receptor or the unknown proteins that couple the receptor to downstream signalling molecules that activate cPLA₂. TNF-induced cytolysis is believed to be mediated through the p55 TNF receptor, leading to the activation of a neutral sphingomyelinase and the generation of ceramide as a second messenger that activates a specific protein phosphatase and protein kinase which, after additional signalling steps, leads to apoptosis (reviewed in references 26 and 38). Most or all of these signalling molecules are associated with membranes and therefore could be targets for E3-10.4K/14.5K. E3-14.7K prevents proteolysis, a crucial early event in TNF-induced apoptosis (79). When the cells are expressing E1A or are treated with CHX, a protease that cleaves the hypothetical protein that blocks TNF activation of cPLA₂ may be activated, thereby allowing cPLA₂ to generate AA and induce cytolysis (Fig. 11). Perhaps E3-14.7K inhibits this protease and in this way prevents TNF activation of cPLA₂. It would be of great interest if this putative protease is a member of the interleukin-1 β -converting enzyme family which is involved in apoptosis (49).

There is a further very interesting implication to our results. The metabolites of AA, the eicosanoids, are potent amplifiers of the inflammatory response (2). To consider the case of an Ad-infected respiratory epithelial cell, TNF-induced synthesis of these eicosanoids could lead to the infiltration of a variety of lymphoid cells capable of killing the infected cell. At the same time, TNF may also activate the Ad E3 promoter and increase synthesis of E3 proteins (39). E3-14.7K and E3-10.4K/14.5K (and perhaps E1B-19K), by blocking TNF activation of cPLA₂,

could prevent an eicosanoid-amplified inflammatory response (Fig. 11). Consistent with this possibility, Sparer et al. (65), studying the lungs of mice infected with Ad mutants with deletions in both E3-14.7K and E3-10.4K/14.5K, found a marked increase in alveolar infiltration of inflammatory cells compared with wild-type Ad. As is the case in cultured cells, the presence of either E3-14.7K or E3-10.4K/14.5K was sufficient to give a wild-type phenotype. In a cotton rat lung model, Ginsberg et al. (16) reported increased infiltration of polymorphonuclear cells in animals infected with a mutant that lacks the E3-10.4K, E3-14.5K, and E3-14.7K proteins. In a vaccinia virus-murine pneumonia model, with the E3-14.7K gene cloned into a vaccinia virus vector that also expresses TNF, E3-14.7K overcame much of the inhibitory effect of TNF on vaccinia virus replication in immunocompetent BALB/c (75) and SCID (76) mice. Thus, E3-14.7K and E3-10.4K/14.5K (and perhaps E1B-19K) may function in Ad infections not only to inhibit TNF cytolysis, thereby allowing Ad replication to occur, but also to inhibit a TNF-induced inflammatory response.

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