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THE ADENOVIRUS E3 REGION 14.7 kDa PROTEIN, HEAT AND SODIUM ARSENITE INHIBIT THE TNF-INDUCED RELEASE OF ARACHIDONIC ACID

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In this report we show that the adenovirus E3 region 14.7 kDa protein, heat and sodium arsenite, which have been defined previously as inhibitors of cytolysis, inhibit the tumor necrosis factor-α (TNF)-induced release of ^{3}H-arachidonic acid from cycloheximide-sensitized C3HA fibroblasts. Since the A23187-induced release of ^{3}H-a.a. was unaffected, our results suggest that these inhibitors provide resistance to lysis by selectively interfering with the lytic response pathway. Our results also show that heat and sodium arsenite can themselves induce the release of ^{3}H-arachidonic acid. These results raise the possibility that stressor-induced resistance to TNF results from the selective desensitization of phospholipase A_2.

The adenovirus E3 region 14.7 kDa protein has been defined as an inhibitor of TNF-induced cytolysis (1, 2). Aside from several negative items of information, i.e., the 14.7 kDa gene product does not affect receptor number or affinity, or the ability of TNF to control MHC gene expression (3), there is no information on the molecular mechanism by which this adenovirus protein provides resistance to TNF. Inducers of the stress response, such as heat and sodium arsenite, have also been shown to inhibit TNF-induced cytolysis (4-6). Again, the information which is available on the mechanism of stressor-induced resistance is largely negative. Initially, it was suggested that the stressor-induced phosphorylation of the small heat shock protein (p28) might mediate stressor-induced resistance to TNF (7). However, since the 28 kDa protein becomes phosphorylated in response to TNF in both sensitive and resistant cells (8), it is unlikely that phosphorylation of this protein is specifically involved in resistance to cytolysis. It is also unlikely that the *de novo* synthesis of stress response proteins is involved in stressor-induced resistance to

ABBREVIATIONS
Tumor necrosis factor-α (TNF), cycloheximide (CHI), ^{3}H-arachidonic acid (^{3}H-a.a.), phospholipase A_2 (PLA_2).

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TNF since resistance can be induced in the presence of inhibitors of transcription and translation (4).

Based on the present understanding of the mechanism of TNF-induced cytolysis it is possible to predict two general modes of action for these inhibitors. Inhibitors of cytolysis could interfere with the expression of the lytic pathway which is activated following the binding of TNF to its receptor. Alternatively, inhibitors of cytolysis could act to protect the cell from the damage with is caused by the lytic pathway, much as manganese superoxide dismutase is envisioned to provide resistance to TNF (9). In this report we have tested the former hypothesis. The activity of cellular phospholipase, which causes the release of arachidonic acid into the culture media at an early stage of the lytic response, has been used as a biochemical marker for the lytic pathway. All the inhibitors we tested, the adenovirus 14.7 kDa protein, heat, and sodium arsenite prevented the TNF-induced release of arachidonic acid suggesting that they provide resistance to cytolysis by interfering with the expression of the lytic response pathway.

MATERIALS AND METHODS

Cells. The cell lines C3HA, C3HA/pBMT (C3HA transfected with and expressing the bovine papilloma virus vector pBMT), and C3HA/p14.7K (C3HA expressing the adenovirus E3 region 14.7 kDa protein in the same vector) have been described previously (3) and were graciously provided to us by L. Gooding, Emory University. These cell lines were grown in Dulbecco's modified Eagles medium supplemented with 10% fetal calf serum and incubated at 37°C in 8% CO_2 .

Reagents. Human rTNF-α and human rIL-1-ß were purchased from Upstate Biotechnology Inc., Lake Placid, NY. CHI, sodium arsenite, and A23187 were purchased from Sigma Chemical Co., St. Louis, MO. All radiolabelled compounds were purchased from New England Nuclear, Boston, MA.

Cytotoxicity Assays. Targets were labelled with 100-200 μCi $Na_2{}^{51}CrO_4$ overnight, harvested by trypsinization, and 1 x 10^4 cells/well were then plated into 96-well flat bottom plates containing TNF with or without CHI. Then, following 16 h of incubation, 100 μl of the supernatant was removed for gamma counting. Percent specific ^{51}Cr release was calculated by the following formula: (experimental - spontaneous release/maximum - spontaneous release) x 100. All points were performed in triplicate and spontaneous release was less that 40% in all experiments.

Arachidonic acid release assays. Cells were plated at 5 x 10^4 cells/well in 12 well flat bottom plates and allowed to adhere 3-4 h before overnight labelling with 0.1 μCi/ml ^{3}H-a.a.((5,6,8,9,11,12,14,15-^{3}H(N)). Cells were then washed twice with Hank's Balanced Salt Solution (HBSS), allowed to incubate for 3 h in media, and washed 2 times with HBSS. Then, following incubation with appropriate reagents, an aliquot of supernatant was removed, centrifuged to remove debris, and counted on a scintillation counter. All points were performed in triplicate and maximal incorporation of label was determined by rigorous scraping of untreated wells.

Stressor Treatments. Heat and sodium arsenite were used to induce resistance to TNF as described previously (4). Cells were either heated at 42.1°C or treated with 75 μM sodium arsenite for 1 h, washed with phosphate buffered saline, and allowed to recover for 3 h in culture media prior to use in ^{51}Cr or ^{3}H-a.a. release assays.

RESULTS AND DISCUSSION

As shown in Fig. 1, C3HA cells are normally resistant to TNF-induced lysis but can be killed by a combination of TNF and CHI. CHI itself, at 10 μg/ml for 16 h is not toxic to these cells. Fig. 1 also shows that C3HA cells transfected with and expressing the bovine papilloma virus vector pBMT remain sensitive to treatment with CHI and TNF while C3HA cells expressing the adenovirus E3 region 14.7 kDa gene product (in the pBMT vector) are almost completely resistant to CHI and TNF. Similarly, treating C3HA cells with 75 μM sodium arsenite for 1 h (followed by a 3 h recovery period) renders them completely resistant to lysis (Fig. 1).

The process of TNF-induced cell death is accompanied by the activation of a cellular phospholipase that causes the release of arachidonic acid (10-13). Inhibitor studies suggest that the enzyme which becomes activated is a form of PLA_2 and that its activity is a necessary component of the lytic response pathway (10-13). The time course of ^{3}H-a.a. release from C3HA cells treated with TNF and CHI is shown in Fig. 2A. Radiolabel release begins typically 2 h after treatment with TNF and CHI is initiated and reaches levels 100-200% above background by the 6 h time point (at which time the release of ^{51}Cr begins). CHI itself also induces a small amount of ^{3}H-a.a. release from C3HA cells, typically 30-50% above background at the 6 h time point. The failure of TNF itself to induce a significant level of ^{3}H-a.a. release has prompted our laboratory to propose that the role of CHI in this system is to inhibit the synthesis of constituitive protein which normally prevents the TNF-induced activation of PLA_2 (10).

As shown in Fig. 2B, expression of the pBMT vector does not alter the overall pattern of ^{3}H-a.a. release from C3HA cells. Again, radiolabel release begins 2 h after treatment with CHI and TNF is initiated and TNF itself remains unable to stimulate the

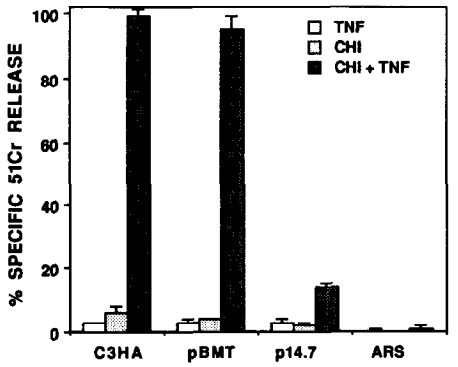

Fig. 1. The results of a typical ^{51}Cr-release assay following treatment with 500 U/ml TNF and/or 10 μg/ml CHI. The cell lines shown are C3HA, C3HA/pBMT (pBMT), C3HA/p14.7K (p14.7), and C3HA treated with sodium arsenite (ARS).

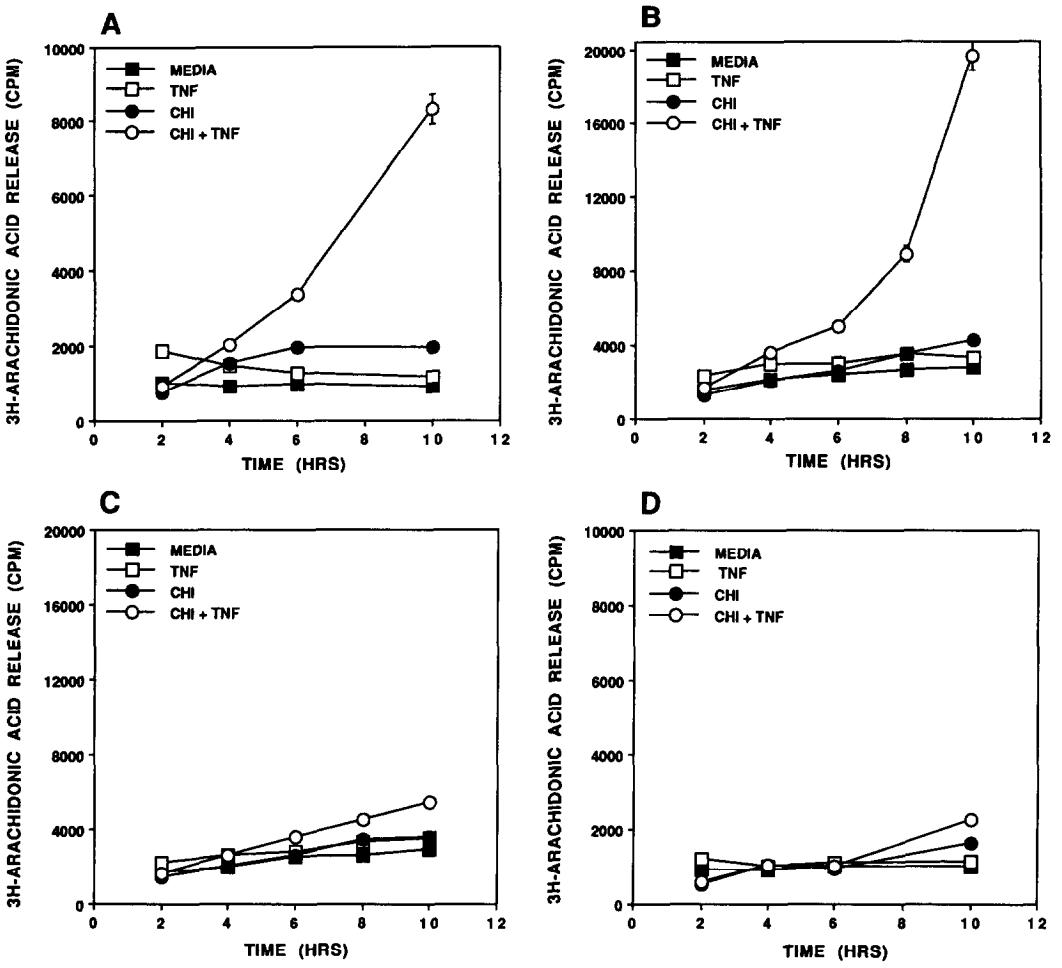

Fig. 2. The release of ^{3}H-a.a. following treatment with 500 U/ml TNF and/or 10 μg/ml CHI. The cell lines shown are C3HA (A), C3HA/pBMT (B), C3HA/p14.7K (C), and C3HA treated with sodium arsenite (D). Results shown are from a single representative experiment.

release of ^{3}H-a.a. Expression of this vector does, however, cause several changes in the responsiveness of C3HA cells. The background level of ^{3}H-a.a. release is increased 1.5-2 fold in most experiments (note the change in scale on the y-axis in Figs. 2B and C) and C3HA cells expressing the vector no longer release ^{3}H-a.a. in response to CHI itself.

As shown in Fig. 2C, expression of the adenovirus E3 14.7 kDa protein almost completely prevents the release of ^{3}H-a.a. in response to CHI and TNF. Furthermore, the effects of the 14.7 kDa protein appear to be selective for the TNF-induced release of ^{3}H-a.a. since expression of the 14.7 kDa protein does not interfere with the ability of CHI and IL-1 to cause the release of ^{3}H-a.a. (Fig. 3A). In fact, expression of the 14.7 kDa appears to stimulate responsiveness to treatment with IL-1 alone. Likewise, while expression of the pBMT vector does suppress responsiveness to A23187, expression of the 14.7 kDa protein

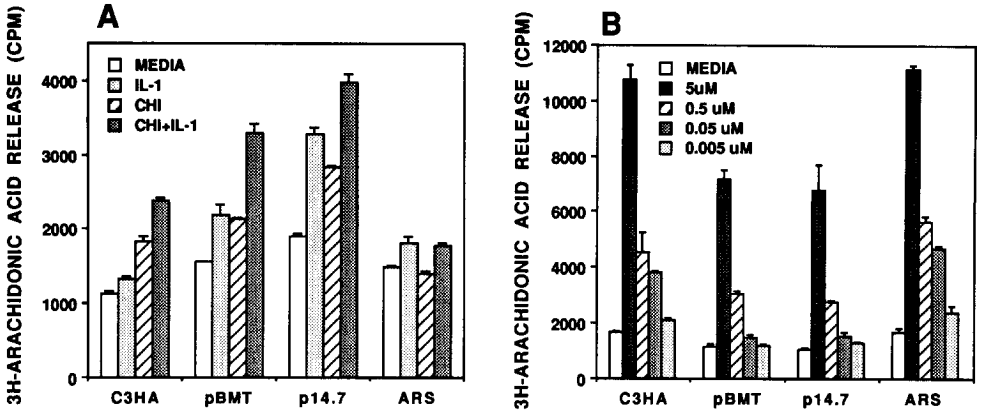

Fig. 3. The release of ^{3}H-a.a. 6 h following treatment with 10 ng/ml IL-1 and/or 10 μg/ml CHI (A) or 1 h after treatment with varying dosages of A23187 (B). The cell lines shown are C3HA, C3HA/pBMT (pBMT), C3HA/p14.7K (p14.7), and C3HA treated with sodium arsenite. Results shown are from a single representative experiment.

does not interfere with the ionophore-induced release of ^{3}H-a.a. (Fig 3B). Overall, these results suggest that the site of action of the 14.7 kDa protein is upstream of the phospholipase and support previous suggestions that this protein is a selective inhibitor of the TNF-induced lytic response pathway (3).

Sodium arsenite also completely suppressed the release of ^{3}H-a.a. from C3HA cells treated with CHI and TNF (Fig. 2D) suggesting that this compound also interferes with the TNF-induced lytic response. In addition, like the 14.7 kDa protein, sodium arsenite did not inhibit the A23187-induced activation of PLA_2 (Fig. 3B). However, unlike the 14.7 kDa protein, sodium arsenite did inhibit the CHI and CHI/IL-1-induced release of ^{3}H-a.a. suggesting that this compound may be a more general inhibitor of PLA_2 activity (Fig 3A).

Previously, it has been reported that heat (another stressor which can induce resistance to TNF) can itself cause activation of PLA_2 and the release of arachidonic acid (14). If heat and sodium arsenite are producing resistance to TNF through the same mechanism, the stimulatory affects of heat would seem to contradict our finding that sodium arsenite can inhibit the release of ^{3}H-a.a. Therefore, to examine this question more closely we measured levels of ^{3}H-a.a. release at each point in the sodium arsenite and TNF/CHI treatment protocol. As shown in Fig. 4B, sodium arsenite does indeed cause the release of ^{3}H-a.a. from C3HA cells. Interestingly, the release of ^{3}H-a.a. occurs during the 3 h recovery period, after the sodium arsenite has been removed.

These results suggest a potential new mechanism for stressor-induced resistance to TNF. The activation of PLA_2 by sodium arsenite may lead to subsequent desensitization of the enzyme, preventing subsequent responsiveness to TNF and CHI, a mechanism which has been suggested for the inhibitory affects of ethanol on the activity of phospholipase C

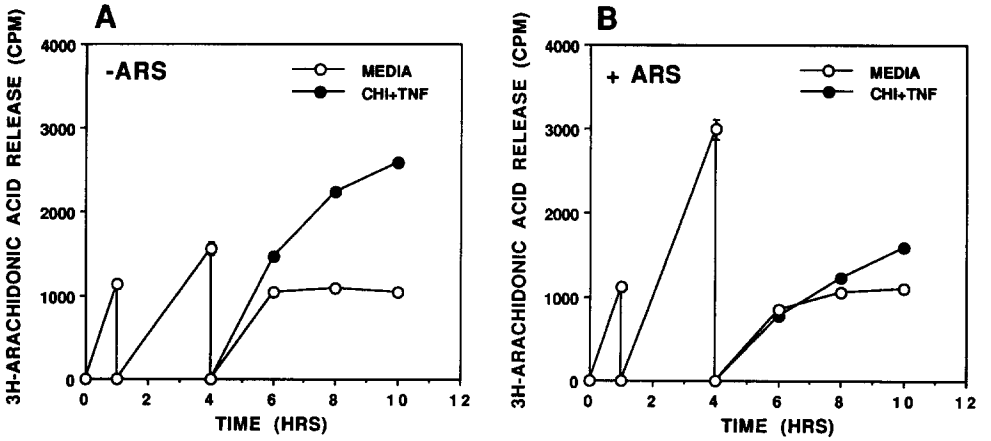

Fig. 4. The release of ^{3}H-a.a. from C3HA cells treated with TNF (500 U/ml) and CHI (10 μg/ml) with either no pretreatment (A) or following pretreatment with sodium arsenite (B). Levels of ^{3}H-a.a. were measured before arsenite treatment, after 1 h with arsenite and after the subsequent wash, at the end of the 3 h recovery period and after the subsequent wash, and then 2, 4, and 6 h after the addition of TNF and CHI. Results shown are from a single representative experiment.

(15). If this hypothesis is correct then heating C3HA cells to 42.1°C for 1 h, followed by a 3 h recovery period (a protocol which has been reported to induce resistance to TNF (4) which we have confirmed) should also induce the release of ^{3}H-a.a. and prevent subsequent response to TNF and CHI. The results of these experiments are shown in Fig. 5. As has been reported previously (14) we find that heat does indeed induce the release of ^{3}H-a.a., and as with sodium arsenite, heat treated cells subsequently failed to release ^{3}H-a.a. in response to TNF and CHI. Unlike sodium arsenite, however, heat treatment did not

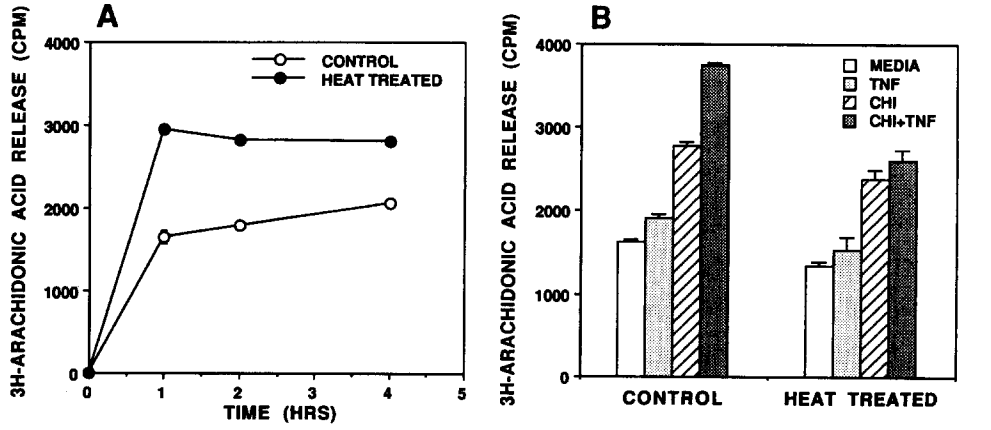

Fig. 5. The release of ^{3}H-a.a. from C3HA cells exposed to 42.1°C for 1 h followed by a 3 h recovery period at 37°C (A). The effect of heat on the subsequent release of ^{3}H-a.a. from C3HA cells treated with 500 U/ml TNF and/or 10 μg/ml CHI (6 h time point) (B). Results shown are from a single representative experiment.

interfere with the CHI-induced release of ^{3}H-a.a. Apparently, the desensitization which is induced by heat is somewhat more selective than that which is induced by sodium arsenite.

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