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# EXTRACELLULAR ATP AND ADENOSINE MODULATE TUMOR NECROSIS FACTOR-INDUCED LYSIS OF L929 CELLS IN THE PRESENCE OF ACTINOMYCIN D

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**Extracellular ATP in concentrations of 0.5 to 2.5 mM modulates TNF-induced cytolysis of L929 cells in the presence of actinomycin D. When present throughout the entire assay period, it inhibits the TNF-induced cytolysis. ADP was less active whereas AMP and GTP were nonreactive. However, inhibition was also achieved by adenosine that was nearly as active as ATP. Yet, the inhibitory effect of ATP was not due to hydrolysis by ectoenzymes to form adenosine. Thus, the nonhydrolyzable ATP analogue adenyl(β-γ-methylendiphosphate) was equally effective in inhibiting TNF-induced cytolysis. Moreover, no conversion of ATP into adenosine was observed during the entire assay period. However, inhibition no longer occurred when the TNF and ATP containing medium was removed after 5 h and replaced by a fresh medium containing TNF and no ATP. We now observed substantial enhancement of the TNF-induced cytolysis by ATP. Finally, treatment with $N^6$-(R-phenylisopropyl)adenosine or with aminophylline, which are thought to downregulate adenosine receptors and to prevent binding of ligands to adenosine receptors, respectively, abolishes adenosine and ATP-mediated inhibition. Again, substantial enhancement of the TNF-induced cytolysis was observed by ATP and only a minor effect by adenosine. The results together suggest that ATP interacts with purinoceptors on the plasma membrane and is capable to enhance and inhibit TNF-induced cytolysis under appropriate conditions. The outcome of the ATP-induced modulation may be influenced by adenosine receptors.**

TNF-α is a cytokine produced mainly by macrophages. It is implicated in diverse biologic processes including cytolysis of certain tumor cells (1). The cytotoxic process is initiated by binding of TNF to high affinity receptors (2–6) and is followed by internalization of TNF receptor complexes (7), increased permeability to cations (8), release of arachidonic acid from cellular phospholipids (9), and degradation and release of nuclear DNA (10). Previous reports have demonstrated that the cytotoxic action of TNF is regulated by various growth factors including IFN-γ (11) and cAMP (12).

Extracellular ATP behaves like a growth factor and acts on cells via receptors that are presumed to reside in the plasma membrane. It stimulates a number of biochemical events that are also induced by well known growth factors. These include increase in the permeability of transformed cells (13), transient elevation of cytosolic $Ca^{2+}$ (13, 14), hydrolysis of phosphatidyl-inositol biphosphate into inositol-1.4.5-triphosphate and diacylglycerol (15), mobilization of free arachidonic acid by activation of phospholipase $A_2$, efflux of $K^+$ through $Ca^{2+}$-activated $K^+$ channels and finally cytoplasmatic alkalinization mediated by a $Na^+$-$H^+$ exchange (16, 17).

The present report shows that extracellular ATP modulates TNF-induced cytolysis of L929 cells by binding to purinoceptors in the plasma membrane.

## MATERIALS AND METHODS

*Cell line.* L929 cells were used as target cells for TNF.

*Medium.* L929 cells were cultured in a medium (culture medium) containing RPMI 1640 (GIBCO, Grand Island, NY; powdered media) supplemented with 10% heat inactivated FCS, HEPES (0.02 M), 2-ME ($4 \times 10^{-5}$ M), and penicillin-streptomycin (109 U/ml).

*Material.* rTNF with an activity of $5 \times 10^7$ U/mg protein was obtained by the courtesy of BASF (Ludwigshafen, FRG). Actinomycin D was obtained from Serva (Heidelberg, FRG). ATP, ADP, GTP, AMP, aminophylline, and $N^6$(R-phenylisopropyl)adenosine were purchased from Sigma Chemical Co. (München, FRG). AMP-PCP[2] was obtained from Boehringer (Mannheim, FRG). The nucleotides and adenosine were dissolved in culture medium. The pH of the solutions were adjusted with NaOH to pH 7.3. [$^{2\text{-}3}$H]adenosine 5′ triphosphate (ammonium salt) 23 Ci/mmol was purchased from Amersham (Frankfurt, FRG).

*Degradation of ATP.* Radioactive ATP (2.5 μCi, 2.5 mM) was added to L929 cell cultures in microtiter plates ($2.5 \times 10^5$ cells/well) and the cultures (2 ml) were incubated at 37°C in the presence or absence of TNF (0.2 μg/ml). After various times 100-μl aliquots were removed and combined with 100 μl 10% trichloracetic acid. The precipitate formed was sedimented in an Eppendorf microfuge. The supernatant (5 μl) was then spotted on cellulose thin layer plates (Macherey-Nagel, Düren, FRG) and the chromatograms were developed with 0.5 M sodium phosphate buffer pH 3.4 (18). Radioactive material was localized by an automatic TCL-linear analyzer (Berthold model LB 2842).

*L929 cytotoxicity assays.* The cytotoxic activity of TNF was determined by a colormetric assay (MTT test) or by the trypan blue exclusion method.

*MTT test (19).* L929 cells were seeded at a density of $5 \times 10^3$ cells/well in 96-well microtiter plates (flat-bottomed) and allowed to incubate for 17 to 24 h in culture medium (200 μl). The supernatant was then removed and replaced by fresh medium containing graded concentrations of TNF (2.5 to 200 ng/ml) and actinomycin D (1 μg/ml). Incubation was then continued and after further 17 h 10 μl of a thiazolylblue solution (5 mg/ml PBS) was then added. After another 4-h incubation, supernatants were removed followed by addition of 150 μl of a HCl/isopropanol solution (40 ml 1 N HCl + 960 ml

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[2] Abbreviation used in this paper: AMP-PCP, adenyl[β,γ-methylen]diphosphate; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5-dimethyltetrazolium bromide.

isopropanol). The absorbance of each well was determined with an automated plate reader (SLT Easy Reader EAR 400 AT) at 550 nm.

*Trypan blue exclusion method.* L929 cells were seeded in Costar (Cambridge, MA) microtiter plates (12 well) at a density of $2.5 \times 10^5$ cells/well and allowed to incubate for 17 to 24 h in culture medium (2 ml). The medium was then removed and replaced with medium containing graded concentrations of TNF and actinomycin D 1 μg/ml. After 17 to 24 h cells were harvested by trypsin/EDTA and the percentage of viable cells was determined employing the trypan blue exclusion method. Per cent cytotoxicity was calculated (= value of test well/value of control well) × 100.

## RESULTS

*Inhibition of TNF-induced cytolysis of L929 cells by ATP.* To determine whether extracellular ATP can affect TNF-induced cytolysis, L929 cells were incubated with ATP in the presence and absence of TNF. Figure 1 shows that ATP (2.5 mM) is capable to inhibit TNF-induced cytolysis of L929 cells. Moreover, Figure 2 shows that this inhibitory effect is dose dependent. Inhibition was observed with as little as 0.5 mM ATP, whereas maximum inhibition required 2.5 mM ATP. At higher concentrations ATP exhibited toxicity (not shown). Inhibition of the TNF-induced cytolysis of L929 cells was also observed with 2.5 mM adenosine whereas ADP was less reactive and GTP and AMP were nonreactive (see Table I).

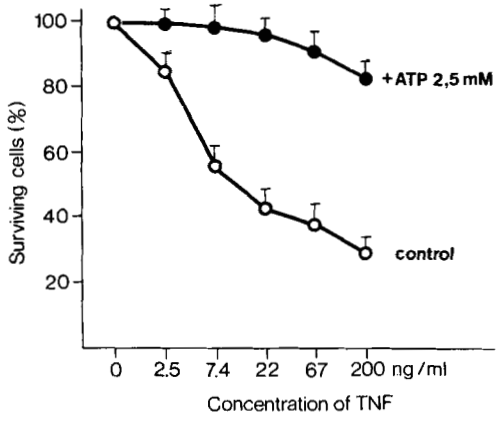


*Figure 1.* Inhibition of the TNF-induced cytolysis of L929 cells by ATP. L929 cell cultures (200 μl) were set up in 96-well microtiter plates ($5 \times 10^3$ cells/well) and permitted to grow overnight. After 17-h cell supernatants were removed and replaced by fresh culture medium. Graded concentrations of TNF were added along with or without ATP (2.5 mM) and actinomycin D (1 μg/ml). Incubation of cultures was then continued and after 24 h surviving cells were determined by the MTT method.

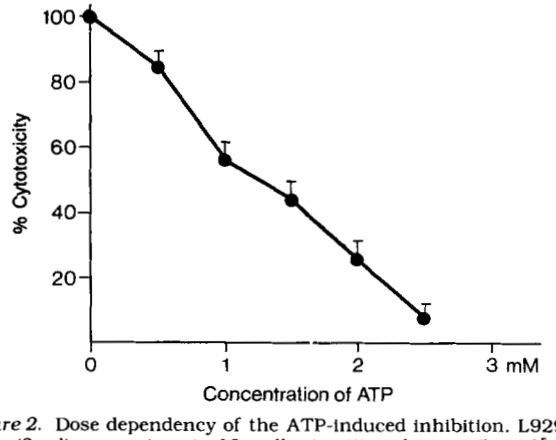


*Figure 2.* Dose dependency of the ATP-induced inhibition. L929 cell cultures (2 ml) were set up in 12-well microtiter plates ($2.5 \times 10^5$ cells/well) as described in the legend to Figure 1. Supernatants were replaced by fresh medium. TNF (100 ng/ml) was then added along with graded concentrations of ATP and actinomycin D (1 μg/ml). Incubation of cultures was then continued and after 24-h surviving cells were determined by the trypan blue exclusion method.

*ATP has to be added together with TNF to achieve maximum inhibition.* To determine whether ATP inhibits early or late events in the TNF-induced cytolysis, it was added to L929 cells before, with or various times after TNF. Preincubation of L929 cells with ATP (2.5 mM) before TNF exposure fails to inhibit cytolysis (data not shown). Moreover, Figure 3 shows that to exert maximum inhibition ATP must be added together with TNF. Addition of ATP 3 or 5 h after TNF exhibits reduced inhibition and 15 h no inhibition.

*Exogenous ATP modulates TNF induced cytolysis without first being converted into adenosine.* The result in the previous section demonstrated that in addition to ATP adenosine inhibits TNF-induced cytolysis of L929 cells. The question, therefore, arose whether ATP was inhibiting cell destruction in its own right or whether it was being converted into adenosine by ectoenzymes present in the plasma membrane. To answer this question, we first measured the conversion of [$^3$H]ATP (2.5 μCi, 2.5 mM) into [$^3$H]adenosine in the presence of L929 cells. After 5 and 24 h no adenosine was detected; all of the tritium was recovered in the medium as unchanged ATP except for 20% conversion to ADP. Moreover, the nonhydrolyzable ATP analogue AMP-PCP was almost as active as ATP in inhibiting TNF-induced cytolysis (Fig. 4).

*Limited exposure of L929 cells to ATP enhances TNF-induced cytolysis.* To determine the time of exposure

TABLE I

*Inhibitory effect of various nucleotides and adenosine on TNF-induced cytolysis of L929 cells*[a]

| Test Substances | Percent Cytotoxicity | |
|---|---|---|
| | Absence of TNF | Presence of TNF 0.2 μg/ml |
| None | | 75.7 ± 8.5 |
| ATP 2.5 mM | 5.0 ± 0.8 | 15.6 ± 2.1 |
| ATP 1.25 mM | 2.5 ± 0.5 | 34.5 ± 5.0 |
| Adenosine 2.5 mM | 3.5 ± 0.39 | 20.5 ± 3.5 |
| Adenosine 1.25 mM | 0.8 ± 0.09 | 64.5 ± 8.1 |
| ADP 2.5 mM | 2.3 ± 0.41 | 30.5 ± 4.1 |
| ADP 1.25 mM | 1.8 ± 0.21 | 50.1 ± 6.1 |
| GTP 2.5 mM | 1.8 ± 0.19 | 72.3 ± 7.0 |
| AMP 2.5 mM | 1.5 ± 0.19 | 74.5 ± 8.9 |

[a] L929 cell cultures (2 ml) were set up in 12-well microtiter plates ($2.5 \times 10^5$ cells/well) and permitted to grow overnight. After 17 h cell supernatants were removed and replaced by fresh medium containing TNF (0.2 μg/ml), nucleotides or adenosine and actinomycin D (1 μg/ml). After further incubation of the cultures (24 h) the cell numbers were determined by trypan blue. The results are the mean ± SEM of four determinations.

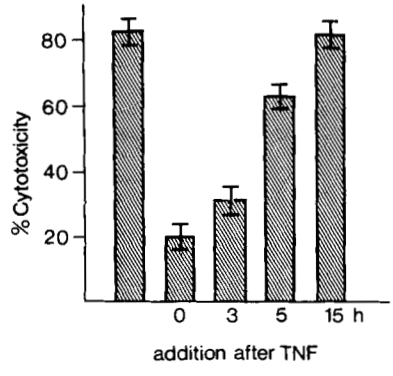


*Figure 3.* Effect of delayed addition of ATP on the inhibition of the TNF-induced cytolysis of L929 cells. Cultures of L929 cells were set up as described in the legend to Figure 2. ATP (final concentration 2.5 mM) was added together with TNF (0.2 μg/ml) and actinomycin D (1 μg/ml) or at the indicated times after TNF. After further incubation (24 h after TNF addition) surviving cells were determined with trypan blue.

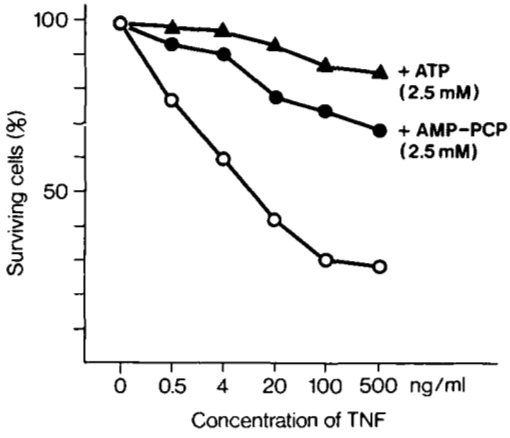


*Figure 4.* Effect of AMP-PCP on the TNF-induced cytolysis of L929 cells. The setting up of the L929 cell cultures, the addition of test substances and the determination of surviving cells were performed as described in the legend to Figure 1.

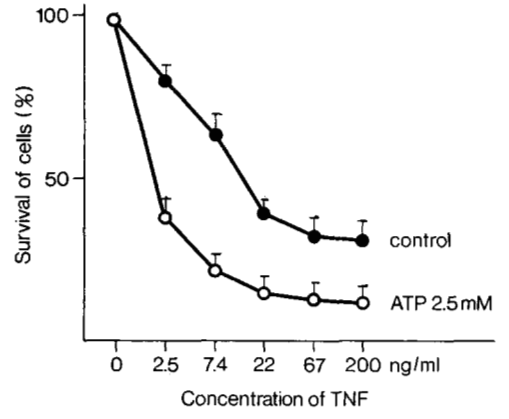


*Figure 5.* Enhancement of the TNF-induced cytolysis of L929 cells by ATP. Cells were cultured in 12-well microtiter plates as described in the legend to Figure 1. Supernatants were replaced by fresh medium. ATP (final concentration 2.5 mM) was added along with graded concentrations of TNF and actinomycin D (1 μg/ml). After 5 h supernatants were washed away and replaced by fresh medium, containing TNF but no ATP. After further incubation (24 h) cell viability was determined by the MTT method.

required to inhibit the TNF response, ATP (2.5 mM) was added together with TNF to a L929 cell culture. After various times, cells were washed and incubation continued in fresh culture medium containing TNF but no ATP. The result demonstrated that ATP has to be present throughout the assay period to be inhibitory (data not shown). Surprisingly, removal of ATP after the first 5 h did not only abolish inhibition: we now observed a marked increase of the TNF-induced cytolysis (Fig. 5). Moreover, pulse experiments in which L929 cells were exposed 2 h to both ATP (2.5 mM) and TNF (0.2 μg/ml) followed by incubation without these agents generated higher cytotoxicity after 24 h (68.5%) than with TNF alone (40.3%).

*Specificity of ATP-induced enhancing effect.* As shown in Table II the enhancing effect of the TNF-mediated cytolysis appears to be specific for ATP. At a concentration of 2.5 mM it enhanced cytotoxicity from 30 to 80%. A similar enhancement was achieved with AMP-PCP, whereas ADP was less reactive and GTP, AMP, and adenosine were nonreactive.

*$N^6$-(R-phenylisopropyl)adenosine and aminophylline abolish inhibitory effect of ATP and unmask its enhancing ability.* Preincubation of L929 cells with $N^6$-(R-phenylisopropyl)adenosine, which have been shown to downregulate adenosine receptors in 3T3 cells (20) prevented adenosine- and ATP-induced inhibition. We now observed a substantial enhancement of the TNF-induced kill by ATP and only a minor effect by adenosine (Table III). Similarly, aminophylline, which was shown to inhibit the binding of adenosine to its receptor, abolished the inhibitory effect of adenosine and converted the inhibitory effect of ATP into an enhancing effect (data not shown). The results together provide indirect evidence that modulation of the TNF-induced cytolysis of L929 cells by ATP is influenced by adenosine receptors.

TABLE II

*Enhancing effects of various nucleotides and adenosine on TNF-induced cytolysis of L929 cells*[a]

| Test Substances | Percent Cytotoxicity | |
|---|---|---|
| | Absence of TNF | Presence of TNF 20 ng/ml |
| None | | 30.2 ± 4.3 |
| ATP 2.5 mM | 2.5 ± 0.31 | 80.0 ± 9.2 |
| AMP-PCP 2.5 mM | 2.4 ± 0.34 | 70.6 ± 6.1 |
| ADP 2.5 mM | 1.5 ± 0.21 | 50.1 ± 7.3 |
| GTP 2.5 mM | 1.8 ± 0.24 | 32.5 ± 4.2 |
| Adenosine 2.5 mM | 2.4 ± 0.31 | 31.4 ± 5.1 |
| AMP 2.5 mM | 1.5 ± 0.21 | 29.3 ± 5.0 |

[a] L929 cell cultures ($2.5 \times 10^5$ cells/well) in 2 ml were set up in 12-well microtiter plates and incubated for 18 h at 37°C. After being washed cells received fresh medium containing the indicated substances, TNF and actinomycin D (1 μg/ml). After further 5 h, supernatants of the cultures were removed and replaced by fresh medium containing TNF, actinomycin D but no test substances. Cultures were then incubated further for another 15-h period. Cell viability was determined with trypan blue. The results are the mean ± SEM of four determinations.

TABLE III

*Effect of R-PIA on modulation of TNF-induced cytolysis of L929 cells by ATP*[a]

| Additions | Percent Cytotoxicity | | | |
|---|---|---|---|---|
| | Alone | +TNF 0.1 μg/ml | +R-PIA 10 μM | +R-PIA + TNF 10 μM 0.2 μg/ml |
| None | | 55.0 ± 4.9 | 8.0 ± 0.9 | 65.3 ± 8.0 |
| ATP 2.5 mM | 10.4 ± 0.9 | 18.3 ± 2.1 | 17.3 ± 2.1 | 90.7 ± 9.0 |
| ATP 1.25 mM | 3.5 ± 0.3 | 35.4 ± 3.5 | 15.7 ± 1.9 | 80.3 ± 8.5 |
| Adenosine 2.5 mM | 2.3 ± 0.2 | 21.2 ± 2.3 | 8.0 ± 0.7 | 65.3 ± 7.2 |
| Adenosine 1.25 mM | 1.9 ± 0.3 | 34.5 ± 4.1 | 7.5 ± 0.5 | 67.5 ± 6.9 |

[a] L929 cell cultures ($2.5 \times 10^5$ cells/2 ml/well) were set up in a 12-well microtiter plate and incubated in the presence or absence of R-PIA (10 μM) for 18 h at 37°C. After being washed, cells received fresh medium containing the indicated test substances and actinomycin D (1 μg/ml). Cells were then incubated further for another 24 h. Cell viability was determined with trypan blue. The results are the mean ± SEM of four determinations; R-PIA; $N^6$(R-phenylisopropyl)adenosine.

## DISCUSSION

The present investigation shows that extracellular ATP is capable to modulate TNF-induced cytolysis of L929 cells in the presence of actinomycin D. It inhibits cytolysis when present throughout the cytotoxic assay but enhances TNF action when present for only the first hours in the assay. ADP was less reactive whereas GTP was nonreactive. The enhancing and inhibitory effects were not due to hydrolysis by ectoenzymes to form adenosine, a known growth factor. Thus, the nonhydrolyzable ATP analogue AMP-PCP is capable to enhance or inhibit TNF-induced cytolysis under the appropriate conditions. In addition, no conversion of ATP into adenosine was observed during the course of the cytotoxic assay.

Furthermore, pretreatment of L929 cells with $N^6$-(R-

phenylisopropyl)adenosine, a known down-regulator of adenosine receptors (20), abolishes inhibition and unveils the ability of ATP to enhance the TNF-induced cytolysis. A similar effect was observed with aminophylline that was shown to prevent binding of ligands to adenosine receptors in 3T3 cells.

ATP inhibits TNF-mediated cytotoxicity by blocking events that occur after TNF binding. This conclusion is based on several observations. Thus, preincubation of L929 cells with ATP before TNF exposure does not alter TNF-induced cytolysis. Moreover, simultaneous addition of ATP and TNF to L929 cells for 2 h followed by incubation without these agents did not block but rather enhanced TNF cytolysis. However, sequential exposure to ATP and then TNF inhibited cytolysis.

The fact that both $N^6$-(R-phenylisopropyl)adenosine and aminophylline abolish ATP-induced inhibition and unmask its enhancing capabilities suggest that TNF-induced cytolysis may be influenced by adenosine receptors. It is feasible that occupancy of the adenosine receptors leads to alterations of the intracellular cAMP levels that are known to influence TNF-induced cytotoxicity. Putative ligands may be present in the culture medium or may be produced and secreted by L929 cells. One possibility is that ATP alone or in combination with TNF triggers the release of endogenously produced ligands that bind to the adenosine receptor and prevent the expression of the lysis program induced by TNF. However, more experiments are needed to distinguish between these possibilities.

The mechanism whereby extracellular ATP enhances TNF-induced cytolysis remains to be determined. Recent experiments have shown that ATP alters ion fluxes in and out L929 cells, induces changes in DAG levels and triggers the release of arachidonic acid from cellular phospholipids (13, 15–17) (V. Lehmann and D. Kinzer, unpublished results). These alterations occur minutes after addition of ATP to L929 cells and are possibly involved in the enhancement of the TNF-induced cytolysis.

The inhibitory and enhancing effect of ATP on the TNF-induced cytolysis is also observed in Wehi and HeLa S cells suggesting that the modulating effects of ATP are more general phenomena.

The results together provide indirect evidence that ATP interacts with purinoceptors on the plasma membrane. It is capable to enhance and to inhibit TNF-induced cytolysis under appropriate conditions. Moreover, the outcome of the ATP-induced modulation may be influenced by adenosine receptors.

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