Secretory nucleoside diphosphate kinases from both intra- and extracellular pathogenic bacteria are functionally indistinguishable

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Nucleoside diphosphate kinase (NDK), responsible for the maintenance of NTP pools, is an ATP-utilizing enzyme secreted by different pathogens. We found that NDK from *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) is also secretory in nature. Secretory NDK is known to play a crucial role in the survival of pathogenic microbes within host cells through their interaction with extracellular ATP. To elucidate this aspect, we assessed the contribution of secretory products containing NDK from intracellular (*Mycobacterium tuberculosis* and *S. Typhimurium*) and extracellular (*Vibrio cholerae*) pathogens to the process of ATP-induced J774 mouse macrophage cell lysis by monitoring lactate dehydrogenase (LDH) release in the culture medium. Compared with an untreated control, our results demonstrate that *S. Typhimurium* secretory products caused a greater than twofold decrease in LDH release from J774 macrophage cells treated with ATP. Furthermore, the secretory products from an *ndk*-deleted strain of *S. Typhimurium* did not display such behaviour. Contrary to this observation, the secretory products containing NDK of *V. cholerae* were found to be cytotoxic to J774 cells. At the amino acid level, the sequences of both the NDKs (*S. Typhimurium* and *V. cholerae*) exhibited 65% identity, and their biochemical characteristics (autophosphorylation and phosphotransfer activities) were indistinguishable. However, to our surprise, the secretory product of an *ndk*-deleted strain of *S. Typhimurium*, when complemented with *V. cholerae ndk*, was able to prevent ATP-induced cytolysis. Taken together, our results unambiguously imply that the intrinsic properties of secretory NDKs are identical in intra- and extracellular pathogens, irrespective of their mode of manifestation.

Received 22 February 2011

Revised 26 July 2011

Accepted 1 August 2011

INTRODUCTION

Host cells infected by pathogens usually accumulate extracellular ATP (eATP) at the site of inflammation as a part of their defence mechanism (Dubyak & el-Moatassim, 1993; Ferrari *et al.*, 1997). The eATP, depending on the duration of exposure to cells, binds to purinergic receptors, particularly P2X7 (Chiozzi *et al.*, 1996; Di Virgilio, 1995), and causes cytolysis via necrotic or apoptotic pathways (Blanchard *et al.*, 1995; Lammas *et al.*, 1997; Molloy *et al.*, 1994; Zaborina *et al.*, 1999a; Zambon *et al.*, 1994). In intracellular pathogens, such as *Mycobacterium tuberculosis*, *Porphyromonas gingivalis* and *Trichinella spiralis*, secretory ATP-utilizing enzymes have been shown to use eATP as

their principal substrate (Chopra *et al.*, 2003; Gounaris *et al.*, 2001; Yilmaz *et al.*, 2008; Zaborina *et al.*, 1999a) and thus prevent host cell death. On the other hand, extracellular pathogens such as *Pseudomonas aeruginosa* and *Vibrio cholerae* have developed strategies to avoid the phagocytic activity of macrophages by releasing enzymes which increase levels of eATP and as a result trigger apoptosis of professional phagocytes (Punj *et al.*, 2000; Zaborina *et al.*, 2000). Thus, it is apparent that the physiological requirements of intracellular and extracellular pathogens are distinct. This has led to the hypothesis that intra- and extracellular pathogens develop strategies in opposite directions (either avoiding or promoting the phagocytic activity of macrophages and/or other professional phagocytes) by releasing secretory ATP-utilizing enzymes which either increase or decrease the level of eATP.

Available literature indicates that secretory enzymes of different pathogens participating in ATP utilization include nucleoside diphosphate kinase (NDK), adenylate kinase, 5' nucleotidase, ecto-ATPase and ecto-nucleoside triphosphate

Abbreviations: Bz-ATP, benzyl-benzyl ATP; eATP, extracellular ATP; GST, glutathione S-transferase; LDH, lactate dehydrogenase; mNDK, *M. tuberculosis* NDK; NDK, nucleoside diphosphate kinase; O-ATP, oxidized ATP; PEI, polyethyleneimine; sNDK, *S. Typhimurium* NDK; vNDK, *V. cholerae* NDK.

Three supplementary figures and a supplementary table are available with the online version of this paper.

diphosphohydrolase (Berrêdo-Pinho *et al.*, 2001; Pinheiro *et al.*, 2006). Among these, NDK is usually a cytoplasmic enzyme, present in virtually all organisms, from prokaryotes to eukaryotes. Beside its predominant involvement in NTP synthesis, NDK performs a variety of functions in bacteria, such as cell growth and differentiation, transcription regulation, site-specific DNA cleavage, and signal transduction (Biggs *et al.*, 1990; Choi *et al.*, 1999; Postel *et al.*, 2000; Steeg *et al.*, 2003). In recent years, extracellular secretion of NDK has been reported in several organisms, including pathogens such as *Leishmania amazonensis* (Kolli *et al.*, 2008), *M. tuberculosis* (Chopra *et al.*, 2003), *P. gingivalis* (Yilmaz *et al.*, 2008), *Pseudomonas aeruginosa* (Zaborina *et al.*, 2000), *T. spiralis* (Gounaris *et al.*, 2001) and *V. cholerae* (Punj *et al.*, 2000). In fact, the involvement of secretory *M. tuberculosis* NDK (mNDK) in phagosome maturation has been shown recently (Sun *et al.*, 2010). However, there is no report available about the secretory nature of *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) NDK (sNDK), which was described as early as 1974 (Ginther & Ingraham, 1974).

Despite being a well-known enzyme, several fundamental questions about bacterial NDKs are still unanswered. In an effort towards understanding this protein in greater detail, we have carried out biochemical characterization of NDKs from intracellular pathogens, *M. tuberculosis* (Tiwari *et al.*, 2004) and *S. Typhimurium* (Dar & Chakraborti, 2010). In this study, the recognition of a concentrated cell-free supernatant of *S. Typhimurium* by anti-mNDK antibody and its ability to synthesize NTPs established the secretory nature of this enzyme. Since NDK utilizes NTPs as its substrate, its secretion from pathogens could be important in the context of eATP metabolism (Dubyak & el-Moatassim, 1993; Ferrari *et al.*, 1997; Zaborina *et al.*, 1999a). We therefore assessed the contribution of secretory NDKs from intracellular (*M. tuberculosis* and *S. Typhimurium*) and extracellular (*V. cholerae*) pathogens to the process of ATP-induced J774 mouse macrophage cell lysis by monitoring lactate dehydrogenase (LDH) release in the culture medium. We show here that the secretory NDK from intracellular pathogens is able to prevent ATP-induced J774 mouse macrophage cell lysis. On the other hand, in an extracellular pathogen such as *V. cholerae*, the concentrated culture supernatant containing *V. cholerae* NDK (vNDK) exhibited a cytotoxic effect on J774 cells. However, the secretory vNDK obtained following its expression in an *ndk*-deleted strain of *S. Typhimurium* (Δ *sndk*) displayed behaviour similar to that of the intracellular pathogens. Thus, our results for what is believed to be the first time provide evidence that secretory NDK possesses ATP-sequestration ability irrespective of its extra- or intracellular mode of manifestation.

METHODS

Materials. Restriction and modifying enzymes were obtained from New England Biolabs. All fine chemicals, including different

nucleotides (ATP, UTP, GTP), oxidized ATP (O-ATP) and benzyl-benzyl ATP (Bz-ATP), were procured from Sigma. The oligonucleotides used in this study were custom-synthesized (Integrated DNA Technologies/Sigma). [γ - 32 P]ATP (~ 5000 Ci mmol $^{-1}$; 185 TBq mmol $^{-1}$) was purchased from Jonaki Laboratories, Board of Radiation and Isotope Technology, Hyderabad, India. Polyethyleneimine (PEI) Cellulose F plates were procured from Merck.

Bacterial strains. *S. Typhimurium* (clinical isolate; Mukherjee *et al.*, 2000), *V. cholerae* (strain N16961) and *M. tuberculosis* (strain H37Ra) were used in this study. Unless mentioned otherwise, all bacterial cultures were grown at 37 °C at ~ 200 r.p.m. in a rotary shaker (Innova 4230, New Brunswick Scientific). For *S. Typhimurium* and *V. cholerae*, Luria-Bertani (LB) broth (Sambrook & Russell, 2001), and for *M. tuberculosis*, Middlebrook 7H9 medium, were used. The bacterial secretory products were prepared as described in earlier studies (Melnikov *et al.*, 2000; Punj *et al.*, 2000; Zaborina *et al.*, 1999b), with minor modifications. Briefly, overnight cultures of *S. Typhimurium* and *V. cholerae* were reinoculated at OD₆₀₀ 0.01, grown for 4 h to exponential phase (OD₆₀₀ 0.4–0.5), harvested and filtered through a 0.22 μ m pore-size filter (Millex-HV, Millipore). Bacterial cells obtained as pellets were, if necessary, resuspended in 50 mM Tris, pH 7.5, containing 150 mM NaCl, and following sonication, the supernatant fraction (also termed 'lysate') was used for determination of intracellular LDH activity. For lag and stationary phase samples of *S. Typhimurium*, cultures were grown in LB to OD₆₀₀ 0.1 and 1.05, respectively. For studies in different media, *S. Typhimurium* was grown in TB (Bacto tryptone, Bacto beef extract), TYE (Bacto tryptone, yeast extract) and M9 (glucose, casamino acids) media to exponential phase (Melnikov *et al.*, 2000). In the case of *M. tuberculosis*, cultures were harvested after 10–12 days of growth. All samples (*S. Typhimurium*, *M. tuberculosis* and *V. cholerae* secretory products) were further concentrated using 10 kDa cut-off concentrators (Amicon) and were confirmed to be free from bacterial contamination.

DNA manipulations. The construction of pET-sNDK (*S. Typhimurium ndk* cloned into the *NdeI/HindIII* sites of pET-28c) has been described previously (Dar & Chakraborti, 2010). *V. cholerae ndk* (*vndk*; locus tag VC0756; Heidelberg *et al.*, 2000) was amplified by PCR from genomic DNA using gene-specific primers (CH3 and CH4, Supplementary Table S1) and cloned into pUC19 at the *SmaI* site to generate pUC-vNDK. This construct was digested with *NdeI/HindIII* and ligated at the corresponding sites of pET-28c to generate pET-vNDK for use in obtaining the recombinant protein (vNDK). A PCR-based overlap-extension method was employed for generation of conserved histidine (H117A) point mutations in both sNDK and vNDK. For this, two external and two internal (incorporating mutation) primers (Supplementary Table S1) were designed. Two sets of primary (pET-sNDK/pET-vNDK as the template) and one set of secondary (mixture of primary reaction products as the template) PCRs were carried out for each mutation. All constructs (wild-type or mutant) were individually transformed into *Escherichia coli* strains DH5 α and BL21(DE3) to increase the amount of DNA and the expression of recombinant proteins, respectively. All mutations were confirmed by sequencing.

Expression and purification of recombinant proteins. *E. coli* BL21(DE3) cells harbouring pET-sNDK, pET-vNDK or histidine mutant constructs were grown overnight in LB broth containing kanamycin (50 μ g ml $^{-1}$). A fresh culture was then set up with 1 % inoculum (from an overnight culture), grown to OD₆₀₀ 0.6 and then induced with 0.4 mM IPTG. Cells after 12 h incubation at 18 °C were harvested, resuspended in lysis buffer [50 mM Tris, pH 7.5, containing 150 mM NaCl, 1 mM PMSF, 1 μ g ml $^{-1}$ each of pepstatin and leupeptin or inhibitor cocktail (Roche)], treated with lysozyme (30 min at 4 °C) and sonicated. The supernatant fraction was further

loaded onto a nickel-nitriloacetate (Ni-NTA) column and washed (10 bed volumes with 50 mM Tris, pH 7.5, containing 150 mM NaCl and 10 mM imidazole), and the purified protein(s) was eluted with elution buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 150 mM imidazole). Following removal of imidazole by dialysis (Dar & Chakraborti, 2010), protein was estimated (Bradford, 1976) and the sample was stored at -80°C until use.

Construction of *ndk* knockout strains. The *ndk* knockout strains of *S. Typhimurium* (Δsndk) and *V. cholerae* (Δvndk) were constructed employing the bacterial conjugation protocol of Miller & Mekalanos (1988). For this purpose, an internal 250 bp fragment of the *sndk* or *vndk* gene was PCR-amplified using pET-sNDK or pET-vNDK as a template with a set of primers (Supplementary Table S1) incorporating *EcoRI/XbaI* sites, and was cloned at the corresponding sites of the pGP704 vector. The construct (pGP704-sNDK250 or pGP704-vNDK250), following transformation into *E. coli* SM102pir (Miller & Mekalanos, 1988; Raychaudhuri *et al.*, 2006), yielded the donor strain. Cultures of the donor and recipient (wild-type *S. Typhimurium* and *V. cholerae*) strains (both OD_{600} 0.4 in LB broth) were mixed, spotted onto an LB agar plate and incubated (16–20 h). The plate was scraped, inoculated into fresh LB broth supplemented with antibiotics (streptomycin and ampicillin, both $100\text{ }\mu\text{g ml}^{-1}$) and kept static overnight (37°C) following vigorous mixing. The culture (upper portion) was plated on an LB agar plate containing the appropriate antibiotics. Colonies were grown for three to four generations, and the *ndk*-deleted strain(s) was confirmed by PCR (data not shown) as well as by Western blotting utilizing anti-mNDK antibody.

pGEX-Kan was constructed by inserting a kanamycin cassette at the *PstI* site of the ampicillin-resistance gene of vector pGEX-KG, and was used for expression of vNDK in Δsndk . For this, pGEX-Kan and pUC-vNDK were digested with *BamHI* and *NdeI*, respectively, treated with Klenow and then digested with *SalI*. The insert and vector following ligation were transformed into DH5 α and selected on LB-kanamycin plates to obtain pGEX-Kan-vNDK. pGEX-Kan-vNDK was transformed into electrocompetent Δsndk cells, and the transformants were selected on LB plates with streptomycin, ampicillin and kanamycin (100 , 50 and $50\text{ }\mu\text{g ml}^{-1}$, respectively). An overnight culture from a single colony was reinoculated in fresh medium, grown to OD_{600} 0.5–0.6, induced with 0.4 mM IPTG (3 h incubation), harvested and processed further. Secretory products from Δsndk , Δvndk and Δsndk transformed with *ndk* of *V. cholerae* or *S. Typhimurium* were prepared as described above (Methods, Bacterial strains).

Autophosphorylation and phosphotransfer assays. The autophosphorylation abilities of sNDK, vNDK and histidine mutants were assessed as described previously (Dar & Chakraborti, 2010; Tiwari *et al.*, 2004). Purified protein (500 ng per reaction) was incubated in $1\times$ kinase buffer with $2\text{ }\mu\text{Ci}$ (74 kBq) [$\gamma\text{-}^{32}\text{P}$]ATP at 24°C for 1 min, and reaction products were resolved on a 12% SDS-PAGE gel. The labelled bands were analysed with a phosphoimager (FLA-9000, Fujifilm) as well as by autoradiography.

The NTP-synthesizing activities of sNDK, vNDK and histidine mutants were monitored in a colorimetric assay performed in microtitre plates (Dar & Chakraborti, 2010; Lasca *et al.*, 1993; Tiwari *et al.*, 2004). Briefly, the enzyme activity of samples (20 ng sNDK or 150 ng vNDK), following incubation with substrate ($5\text{--}400\text{ }\mu\text{M}$ ADP) and other reaction ingredients (reaction volume= $50\text{ }\mu\text{l}$), was monitored at 490 nm in an ELISA plate reader (SpectraMax Plus 384, Molecular Devices). The values obtained were corrected by subtracting the blank readings (no significant difference was noticed when assays were carried out with all ingredients, except either substrate or sNDK and dGTP). Standard curves were prepared with ATP ($5\text{--}200\text{ }\mu\text{M}$; omitting dGTP, ADP and sNDK or vNDK in reaction mixtures), and the activity of sNDK/vNDK was expressed as $\mu\text{mole ATP produced min}^{-1}(\text{mg protein})^{-1}$.

To evaluate the NDK activity in the cell-free supernatant, a kinase assay was performed using PEI TLC plates (PEI-TLC Cellulose F plates, 1.05579.0001, Merck). Briefly, $5\text{ }\mu\text{l}$ concentrated cell-free supernatant containing $5\text{ }\mu\text{g}$ total protein was incubated with $2\text{ }\mu\text{Ci}$ (74 kBq) [$\gamma\text{-}^{32}\text{P}$]ATP, 1 mM NDP or a mixture of NDPs and 1 mM MgCl_2 (Melnikov *et al.*, 2000; Kolli *et al.*, 2008). The reaction was performed at room temperature for 10 min and then stopped by incubating at 4°C (over ice). Finally, $2.5\text{ }\mu\text{l}$ of the reaction product was separated on a PEI-TLC plate using 0.75 M KH_2PO_4 buffer (pH 3.5). The plate was air-dried, developed and analysed on a phosphoimager as well as by autoradiography. For adenylate kinase activity, the concentrated cell-free supernatant was incubated with [$\gamma\text{-}^{32}\text{P}$]ATP in the presence of 1 mM AMP (Melnikov *et al.*, 2000; Kolli *et al.*, 2008).

Macrophage culture and LDH assay. J774 mouse macrophage cells ($3\text{--}5\times 10^6$) in 10 ml RPMI medium with 10% (v/v) fetal calf serum (FCS) were cultured in a 90 mm tissue culture plate at 37°C in a CO_2 incubator (95% $\text{O}_2/5\text{ }\%$ CO_2) for 16–18 h. Cells were harvested (2000 g for 2–3 min at 4°C), washed three times with chilled PBS and then counted using the trypan blue method. Cells (2×10^5 per well) in RPMI with 3% FCS were seeded in 24-well plates and incubated at 37°C for 2–3 h to test adherence. Following washing with PBS, adhered cells were incubated in RPMI/10% FCS with ATP or concentrated cell-free filtrate containing bacterial secretory products (*S. Typhimurium*, *M. tuberculosis*, *V. cholerae*, Δsndk , Δvndk or Δsndk transformed with *ndk* of *V. cholerae* or *S. Typhimurium*) with or without ATP for 4 h (Melnikov *et al.*, 2000; Kolli *et al.*, 2008; Zaborina *et al.*, 1999b). In all cases the effect of bacterial secretory products was evaluated using 1, 5 or $10\text{ }\mu\text{g}$ total protein (in $10\text{ }\mu\text{l}$), except for *M. tuberculosis*, where 1, 5 or $10\text{ }\mu\text{l}$ of concentrated sample (processed similarly) was used. At the end of incubation, the supernatant ($50\text{ }\mu\text{l}$) was removed and the LDH activity (of the supernatant) was colorimetrically determined with a CytoTox 96(R) Non-Radioactive Cytotoxicity Assay kit, following the manufacturer's protocols (Promega). The LDH activity obtained in the supernatant ($50\text{ }\mu\text{l}$) of cells without any treatment served as a blank. The blank value was subtracted in each case, and the percentage LDH release was calculated taking $0.2\text{ }\%$ Triton X-100-lysed cells as 100% (Kolli *et al.*, 2008).

Western blotting. Samples ($\sim 10\text{ }\mu\text{g}$ per slot) of wild-type (*S. Typhimurium* or *V. cholerae*), their *ndk*-deleted strains, or concentrated cell-free secretory products, after resolving by 12% SDS-PAGE, were transferred (120 V for 1 h) to a nitrocellulose membrane ($0.45\text{ }\mu\text{m}$) using Tris-glycine-SDS buffer (Dar & Chakraborti, 2010). Blots were incubated in primary (anti-mNDK) and secondary (anti-rabbit IgG) antibodies, or horseradish peroxidase-conjugated anti-glutathione S-transferase (GST) antibody, and developed with the ECL detection system (GE Healthcare) following the manufacturer's recommended protocol.

Bioinformatic analysis. The multiple sequence alignment of the protein sequences retrieved from the mail server at the NIH, USA (<http://www.ncbi.nlm.nih.gov>), was carried out using the CLUSTAL_X 1.81 program (Thompson *et al.*, 1997).

Statistical analysis. Data were analysed with Student's *t* test, the non-parametric Mann-Whitney two-tailed test and repeated-measure analysis of variance with the Student-Newman-Keuls multiple-comparisons post test using InStat software (version 3, GraphPad).

RESULTS

sNDK is secretory in nature

NDKs from different bacteria have been shown to be secretory in nature (Chopra *et al.*, 2003; Punj *et al.*, 2000;

Yilmaz *et al.*, 2008; Zaborina *et al.*, 2000). To elucidate this aspect in *S. Typhimurium*, concentrated culture supernatant from different phases of bacterial growth was incubated with NDPs (CDP, GDP and UDP) in the presence of [γ - 32 P]ATP, and the reaction products were resolved on TLC plates (see Methods). This led to the formation of the corresponding NTPs (CTP, GTP and UTP) in the exponential phase of the culture (Fig. 1a, left panel and Supplementary Fig. S1a), suggesting NDK-like activity in the samples. Among the different culture media tested (LB, TB, TYE and M9), such an activity could not be observed in M9 medium (Supplementary Fig. S1b). In subsequent studies, we used exponential phase cultures of *S. Typhimurium* grown in LB medium. The same experiment, when carried out with concentrated culture supernatant of *M. tuberculosis*, yielded identical results (Fig. 1a, right panel, compare lane 1 with lanes 2, 3 and 4). We carried out Western blotting using anti-mNDK antibody (Tiwari *et al.*, 2004). As shown in Fig. 1(b), this antibody was able to recognize sNDK as well (upper panel, lane 2). Further, the cell lysate and concentrated cell-free supernatant prepared from exponential phase culture (see Methods) of *S. Typhimurium* were resolved by SDS-PAGE and subjected to immunoblotting using anti-mNDK antibody. Interestingly, in both cell lysate and concentrated cell-free supernatant of *S. Typhimurium*, bands corresponding to the expected size of NDK (~16.5 kDa) were highlighted (Fig. 1c, upper panel, lane 3). To rule out the possibility of any bacterial contamination, the concentrated cell-free supernatant was spotted onto LB agar without any antibiotic selection. As expected, the concentrated cell-free supernatant did not show such contamination (Fig. 1d, upper panel, lane 3). To make sure that the presence of NDK in concentrated cell-free supernatant was not due to cell lysis, we determined its LDH activity. LDH is a known cytoplasmic enzyme and is used as a marker for cell lysis. As shown in Fig. 1(d) (lower panel), only cell lysate (supernatant prepared from sonicated cells) exhibited LDH activity [compare panels of lysate versus concentrated cell-free supernatant and also concentrated medium (inset)]. Thus our results argue that the secretory product of *S. Typhimurium* very likely contains NDK.

Since NDK utilizes ATP as its substrate, its secretion from pathogens could be important in the context of ATP sequestration, and as a result it should prevent cytolysis (Gounaris *et al.*, 2001; Kolli *et al.*, 2008; Yilmaz *et al.*, 2008). Based on this hypothesis, we utilized an assay system in which the cytotoxic effect or behaviour of ATP was studied by monitoring the release of the cytoplasmic marker enzyme LDH from J774 cells (Kolli *et al.*, 2008; Melnikov *et al.*, 2000; Punj *et al.*, 2000). The system displayed ATP-dependent release of LDH from J774 cells (Fig. 2a, compare first three bars), and this effect was ATP-specific, as treatment with either GTP or UTP did not cause any LDH release (Fig. 2a and inset, showing that the percentage LDH release was equal to that from cells without any treatment). To test whether ATP-induced

LDH release is mediated only through P2X7 receptors, J774 macrophage cells were pre-treated with a known receptor-specific antagonist and an antagonist (Melnikov *et al.*, 2000; Punj *et al.*, 2000; Yilmaz *et al.*, 2008). As expected, while pre-treatment (1 h) with the irreversible antagonist O-ATP (0.3 mM) prevented ATP-induced LDH release, an agonist, Bz-ATP (2 mM), did not affect such activity (Fig. 2a, compare last two bars). In our experimental conditions, we observed ~60 % LDH release in response to ATP treatment of J774 macrophage cells, which is well within the range (20–75 %) reported by others (Kolli *et al.*, 2008; Melnikov *et al.*, 2000). Compared with treatment with ATP alone, incubation of concentrated cell-free supernatants from either *M. tuberculosis* or *S. Typhimurium* with adhered J774 cells in the presence of ATP prevented LDH release in a dose-dependent manner (Fig. 2b). This was an ATP-dependent phenomenon, since the percentage LDH release in response to concentrated cell-free supernatants from either *M. tuberculosis* or *S. Typhimurium* was similar to that of the cells without any treatment (inset of Fig. 2b). Since 10 $\times$ concentrated cell-free supernatants displayed maximum prevention of ATP-induced J774 cell death (in the presence of 3 mM ATP, percentage LDH release with *S. Typhimurium* was 17.98 ± 3.94 and with *M. tuberculosis* = 23.7 ± 4.88 , as opposed to 58.9 ± 5.45 with ATP alone; $n=5$ in all cases; $P<0.001$; Fig. 2b), in subsequent assays this amount was used. Thus our results indicated that secretory ATP-utilizing enzymes, including NDK of intracellular pathogens such as *M. tuberculosis* and *S. Typhimurium*, could prevent the ATP-induced cytotoxicity of J774 macrophage cells.

We further constructed an *ndk* knockout strain of *S. Typhimurium* (Δ *ndk*) employing a bacterial conjugation method (see Methods and Supplementary Fig. S2a). The deletion of NDK was confirmed by Western blotting using anti-mNDK antibody (Supplementary Fig. S2b). To substantiate that the NTP-synthesizing activity of the cell-free supernatant of *S. Typhimurium* was due to the presence of NDK, the secretory product from the Δ *ndk* strain was incubated with [γ - 32 P]ATP and a mixture of NDPs. Fig. 3(a) categorically demonstrates that the secretory product (concentrated) obtained from the culture of the Δ *ndk* strain was unable to synthesize any of the NTPs compared with that of the wild-type (left panel, lane 3). Purified protein (sNDK) was included as a positive control in this experiment, which on incubation with mixture of NDPs (CDP, GDP and UDP) efficiently converted them to the corresponding NTPs (Fig. 3a, left panel, lane 4). Interestingly, concentrated cell-free supernatant from an *ndk*-deleted strain of *S. Typhimurium* on incubation with [γ - 32 P]ATP and AMP was able to form labelled ADP but unable to synthesize ATP, suggesting that deletion of NDK did not affect the adenylate kinase-like activity of the secretory product from the pathogen (Fig. 3a, right panel, compare lanes 2 and 3). The formation of labelled products by the Δ *ndk* strain in the presence of [γ - 32 P]ATP (Fig. 3a, left panel, lane 3) may be due to the

Fig. 1. Secretory nature of *S. Typhimurium* NDK. (a) NTP-synthesizing ability of cell-free culture supernatant from *S. Typhimurium* or *M. tuberculosis*. The concentrated supernatant from the culture of *S. Typhimurium* (S.t) or *M. tuberculosis* (M.t) was incubated with [γ - 32 P]ATP in the presence of NDPs as indicated and then resolved on TLC plates. The reaction products were visualized in a phosphorimaging device and by autoradiography. The positions of the different NTPs synthesized are indicated by arrows. (b) Western blotting of recombinant NDK from *S. Typhimurium* (sNDK) using anti-mNDK antibody (upper panel). A Coomassie brilliant blue-stained gel of recombinant NDKs served as loading control (lower panel). (c) Detection of NDK in secretory products of *S. Typhimurium*. Samples [total cell lysate (Cell lysate) and concentrated secretory product (Conc. Supernatant S.t)] were resolved by SDS-PAGE and immunoblotted using anti-mNDK (upper panel) or stained with Coomassie brilliant blue (lower panel). Concentrated medium alone (lane 2) served as a negative control. (d) Samples (5 μ l each) of exponential phase culture supernatant of *S. Typhimurium* (ST) before filtration (BF), after filtration (AF) and concentrated (CONC) were spotted on LB agar plates without any antibiotic selection (upper panel). Lower panel: LDH activity in supernatant of cells from an exponential phase culture of *S. Typhimurium* following sonication and in the concentrated cell-free supernatant (after filtration) (Conc. supernatant) of the same culture. Inset: LDH activity of Tris-NaCl buffer, pH 7.5 (used for resuspending *S. Typhimurium* cells) (Buffer), and concentrated LB broth (Conc. medium).

combined action of adenylate kinase and 5'-nucleotidase enzyme activities present in the cell-free supernatant of the *ndk*-deleted strain of *S. Typhimurium*. Similar behaviour has been reported earlier in *Burkholderia cepacia* (Melnikov *et al.*, 2000) and *Pseudomonas aeruginosa* (Zaborina *et al.*, 1999b), in which in the absence of any substrate, the

incubation of the secreted product with [γ - 32 P]ATP results in the formation of labelled products, including ADP.

We also tested the effect of deletion of *ndk* on the ATP-scavenging ability of *S. Typhimurium* concentrated culture supernatant. For this, J774 macrophage cells were treated

Fig. 2. Role of secretory NDK in ATP-induced J774 macrophage cell lysis. (a) Effect of ATP on J774 cells. Macrophage cells were treated with different concentrations of ATP (1, 3 and 5 mM) or different NTPs (GTP, UTP; 3 mM each) or pre-treated for 1 h with O-ATP (0.3 mM) or Bz-ATP (2 mM), and then incubated with ATP (3 mM) for 4 h as indicated. Samples were collected as described in the text and analysed for LDH release. The amount of LDH released by cells alone served as a blank and was subtracted from each sample. Inset: LDH release by different NTPs (3 mM) compared with that due to cells alone. (b) Effect of secretory NDKs from intracellular pathogens on the modulation of ATP-induced cell lysis. Macrophage cells were incubated with buffer or ATP for 4 h in the presence or absence of different doses (1×, 5× and 10×) of concentrated cell-free supernatant from *S. Typhimurium* (S.t) or *M. tuberculosis* (M.t) as indicated, and an LDH release assay was performed. Percentage LDH release was calculated taking 0.2% Triton X-100-lysed cells as 100%. Cells alone served as a blank and this value was subtracted from test samples. Data represent mean and SD ($n=3$ or 5). ** and *** indicate $P<0.01$ and $P<0.001$, respectively; 1×, 5× and 10× represent 1, 5 and 10 μ g for *S. Typhimurium*, and 1, 5 and 10 μ l for *M. tuberculosis*. Inset: LDH release from cells in response to no treatment (cells only) or treatment with 10× concentrated culture supernatant from *S. Typhimurium* (S.t) or *M. tuberculosis* (M.t) in the absence of ATP.

with concentrated cell-free supernatant of the Δ sndk strain, alone or in the presence of ATP, and cell lysis was assessed by monitoring LDH release. Interestingly, we found that the ATP was able to cause significant ($P<0.001$) J774 cytotoxicity when incubated with concentrated culture supernatant from the Δ sndk strain (percentage LDH release in Δ sndk + ATP = 41 ± 6.2 , $n=3$; Fig. 3b). As expected, the percentage LDH release with the Δ sndk strain was very close to that of cells without any treatment (percentage LDH release in Δ sndk = 19.2 ± 3.2 , as opposed to 18.5 ± 3.6 for cells alone; $n=3$ in each case; inset of Fig. 3b). Thus these results indicate that the prevention or modulation of ATP-induced cell lysis by secretory ATP-utilizing enzymes of *S. Typhimurium* is mediated through NDK.

Secretory vNDK is cytotoxic to J774 mouse macrophage cells

To have an insight into the secretory nature of the NDK of the extracellular pathogen *V. cholerae*, cell lysate and concentrated culture supernatant were prepared from

exponential phase cultures (see Methods). Samples, after resolving by SDS-PAGE, were subjected to Western blotting using anti-mNDK antibody. The antibody bound to bands at ~15.8 kDa (Fig. 4a), which was the expected size of vNDK. TLC of the concentrated culture supernatant upon incubation with different NDPs in the presence of [γ - 32 P]ATP yielded the corresponding NTP, which was suggestive of an NDK-like activity in the samples (Fig. 4b).

Further, we observed that incubation of concentrated cell-free supernatants from *V. cholerae* with adhered J774 cells in the presence of ATP did not prevent LDH release. In fact, there was hardly any difference in the level of LDH released in the presence of ATP alone (percentage LDH release 56.8 ± 7.7 , $n=3$) or ATP and secretory product (65.3 ± 4.7 , $n=3$; Fig. 4c). *V. cholerae* secretory product containing NDK (vNDK), even in the absence of ATP, displayed a cytotoxic effect (percentage LDH release 63.25 ± 6.7 , $n=3$; Fig. 4c). This was not an experimental artefact, since the cytotoxicity rendered by concentrated cell-free supernatants of *V. cholerae* increased in a

Fig. 3. Characterization of the *ndk*-deleted strain of *S. Typhimurium*. (a) NTP-synthesizing ability of Δ *sndk*. Concentrated cell-free culture supernatant from wild-type (*S.t*) or Δ *sndk* was incubated with [γ -³²P]ATP and a mixture of NDPs (left panel) or AMP (right panel) in the presence of Mg²⁺ (1 mM) for 10 min. Reaction products were separated by TLC and visualized in a phosphoimaging device and by autoradiography. The positions of the labelled products formed are shown by arrows. P.P, purified recombinant NDK protein from *S. Typhimurium* included as a control. (b) Effect of *ndk* deletion on ATP-induced cell lysis. J774 cells were incubated with or without ATP for 4 h in the presence or absence of concentrated cell-free culture supernatant from the Δ *sndk* strain, and LDH release was assessed. Results are means and SD ($n=3$) and represent the amount of LDH released after subtracting the blank (cells only) readings. ***, $P<0.001$. Inset: LDH release from cells in response to no treatment (cells only) or treatments with 10× concentrated culture supernatant from the Δ *sndk* strain in the absence of ATP.

dose-dependent manner (inset, Fig. 4c, compare LDH release of 1 × and 10 × concentrated culture supernatant). Thus our results are in conformity with those of others (Punj *et al.*, 2000; Zaborina *et al.*, 2000) and support the

notion that secretory products containing ATP-utilizing enzymes, including NDKs of extracellular pathogens, are cytotoxic to macrophages. It is interesting to mention here that the secretory product containing ATP-utilizing enzymes (including NDK) of a *V. cholerae* toxin-deficient strain (*V. cholerae* VB1) exhibits similar behaviour (Punj *et al.*, 2000).

NDKs from *S. Typhimurium* and *V. cholerae* exhibit identical biochemical characteristics

NDK, one of the secretory ATP-utilizing enzymes, is involved in preventing ATP-induced cell lysis (Fig. 2) in intracellular pathogens (*S. Typhimurium* and *M. tuberculosis*), while its extracellular counterpart (*V. cholerae*) is cytotoxic to J774 mouse macrophage cells (Fig. 4). Any alteration in protein functionality is likely to be reflected in the sequence. Interestingly, a pairwise sequence comparison indicated that sNDK and vNDK share 65% identity (Supplementary Fig. S3a). NDK is known to maintain the NTP/dNTP pools of a cell by synthesizing different NTPs/dNTPs through a high-energy phosphorylated intermediate (Lacombe *et al.*, 2000; Lascu, 2000). Both sNDK and vNDK recombinant proteins were found to display autophosphorylation as well as phosphotransfer activities (Fig. 5a, b). NDK is known to be a histidine kinase, in which a highly conserved active site histidine residue is indispensable for the activity of the protein. To determine the effect of a mutation on the activities of the proteins, His-117 was mutated to alanine, and the protein was then assessed for autophosphorylation and phosphotransfer ability. The results demonstrated that the mutation abolished both the activities of the recombinant proteins, making them kinetically inactive (Fig. 5a, b). Kinetically these proteins also showed minor differences, e.g. the enzyme turnover rate of sNDK was slightly higher compared with vNDK (Table 1). Interestingly, recombinant NDK from another intracellular pathogen, *M. tuberculosis*, exhibited an approximately ninefold increase in enzyme turnover rate compared with vNDK (Tiwari *et al.*, 2004).

Secretory NDKs from *S. Typhimurium* and *V. cholerae* exhibit identical behaviour

Despite their highly homologous amino acid sequences and similar biochemical properties (Fig. 5), the distinction in the functionality of secretory NDKs from extra- or intracellular pathogens in preventing ATP-mediated cytotoxicity (Figs 2 and 4) was surprising. To account for the bifunctional nature of this secretory protein, we expressed NDK from *V. cholerae* in an *ndk*-deleted strain of *S. Typhimurium*. Fig. 6(a) clearly shows the expression of an ~44 kDa induced band, which corresponds to the expected size of the protein of interest (compare lanes 1–5 with lane 6), and its authenticity as vNDK was confirmed by Western blotting using anti-mNDK (Fig. 6a, middle panel) and anti-GST (Fig. 6a, lower panel) antibodies.

Fig. 4. Role of *V. cholerae* NDK in ATP-induced cell lysis. (a) Western blotting of cell lysate and concentrated cell-free culture supernatant (Conc. supernatant) from *V. cholerae* using anti-mNDK antibody. Samples from *V. cholerae* were prepared, resolved by SDS-PAGE and transferred to nitrocellulose membranes (upper panel) or stained with Coomassie brilliant blue (lower panel). The blot was probed with anti-mNDK antibody. Lanes: 1, cell lysate; 2, concentrated medium (LB); 3, concentrated cell-free supernatant of *V. cholerae* (upper/lower panel). (b) NTP-synthesizing ability of cell-free supernatant from *V. cholerae*. The concentrated supernatant from *V. cholerae* culture (V.c) was incubated with [γ - 32 P]ATP in the presence of different NDPs and then resolved by TLC. The reaction products were visualized in a phosphoimaging device and by autoradiography. The positions of different NTPs synthesized are indicated by arrows. P.P, purified protein included as a control. (c) Effect of secretory NDK on the modulation of ATP-induced cell lysis. J774 cells were incubated with buffer or ATP for 4 h in the presence or absence of concentrated cell-free culture supernatant of *V. cholerae* (V.c). An LDH release assay was performed after incubation. Data represent mean and SD ($n=3$). The LDH release of cells alone (blank) was subtracted from all test samples. Inset: LDH release from cells in response to no treatment (cells only) or treatments with 1 $\times$ or 10 $\times$ concentrated culture supernatant from *V. cholerae* (V.c) in the absence of ATP (1 $\times$ and 10 $\times$ represent 0.01 and 0.1 μ g protein, respectively).

To test whether the expression of NDK from an extracellular pathogen (*V. cholerae*) could rescue the NTP-synthesizing deficiency of an *ndk*-deleted strain of *S. Typhimurium*, concentrated culture supernatant from a vNDK-transformed Δ *ndk* strain was prepared and analysed for NTP synthesis. The results obtained clearly demonstrated that expression of NDK from an extracellular pathogen can complement the function of an intracellular pathogen by restoring the NTP-synthesizing ability of the Δ *ndk* strain (Fig. 6b, compare lanes 4 and 5 with lanes 6 and 7). As expected, the vector transformed in Δ *ndk* was unable to synthesize any NTP (Fig. 6b, compare lanes 6 and 7 with lanes 8 and 9). We also monitored LDH release following incubation of concentrated culture supernatants (10 μ g protein) of the Δ *ndk* strain, complemented

with either vNDK or sNDK, with J774 cells in the presence of ATP. Surprisingly, we observed that incubation with concentrated culture supernatant from the vNDK-complemented Δ *ndk* strain was able to prevent ATP-induced LDH release from J774 cells (21.9 ± 4.05 %, compared with 55.85 ± 2.0 % with ATP alone, $n=5$; Fig. 6(c), compare bars 1 and 4). In fact, the behaviour of the wild-type strain of *S. Typhimurium* and the Δ *ndk* strain complemented with either sNDK or vNDK was indistinguishable in the presence (Fig. 6c, compare bars 2 and 3 with bar 4) or absence (inset, Fig. 6c) of ATP. Furthermore, the concentrated secretory product from the *ndk*-deleted strain of *V. cholerae*, when incubated with J774 cells, exhibited cytotoxic behaviour even in the absence of ATP (Fig. 6c). Thus all these lines of evidence unambiguously rule out the

Fig. 5. Biochemical characteristics of recombinant NDKs. (a) Autophosphorylation activity of recombinant purified proteins. Wild-type (sNDK or vNDK) or mutant proteins (500 ng per reaction) were incubated with [γ - 32 P]ATP in the presence of Mg^{2+} for 1 min. Samples were resolved by SDS-PAGE and analysed in a phosphoimaging device and by autoradiography. A Coomassie brilliant blue-stained gel served as a loading control. The positions of molecular mass markers (in kDa) are indicated. (b) Phosphotransfer activity. ATP formation by vNDK (150 ng per reaction) was determined by using dGTP as donor and ADP as acceptor nucleotide, respectively, and the amount of ATP generated per minute was calculated as described in Methods. Results are mean and SD ($n=3$). The difference in phosphotransfer activities between wild-type and mutant proteins is significant ($P<0.001$).

possibility of any distinction in the function of NDKs between extracellular and intracellular pathogens.

DISCUSSION

NDK is a highly conserved ubiquitous enzyme primarily responsible for maintaining nucleotide pools in micro-organisms. Autophosphorylation of the protein occurs as a part of its catalytic mechanism, which leads to the phosphotransfer reaction from NTP/dNTP to NDP through a high-energy phosphorylated intermediate (Lacombe *et al.*, 2000). Since NDK has very little specificity for the base or the sugar, it has the ability to accept any NTP/dNTP as its substrate. A growing body of evidence now indicates that NDKs, as well as other ATP-utilizing enzymes of microbes, especially in pathogens, are secretory in nature and involved in host–pathogen interactions. In fact, host cells as a part of their defence strategy, when infected by pathogens, accumulate eATP at the site of inflammation, which causes cell death (Dubyak & el-Moatassim, 1993; Ferrari *et al.*, 1997). It has now been postulated that secretory NDK or other ATP-utilizing enzymes from pathogens modulate the level of eATP and thus exploit the situation in favour of their survival (Chopra *et al.*, 2003; Gounaris *et al.*, 2001; Yilmaz *et al.*, 2008; Zaborina *et al.*, 1999a). However, the distinct physiological requirements of intra- and extracellular pathogens complicate the situation slightly. This led to the hypothesis that intra- and extracellular pathogens use secreted ATP-utilizing enzymes in opposite directions, by either avoiding or promoting the phagocytic activity of macrophages and/or other professional phagocytes (Kolli *et al.*, 2008; Punj *et al.*, 2000; Yilmaz *et al.*, 2008; Zaborina *et al.*, 2000). To rationalize the notion, we carried out our studies with intracellular (*S. Typhimurium*) and extracellular (*V. cholerae*) pathogens to explore the behaviour of secretory NDK.

NDK is reported to be the only enzyme involved in the maintenance of NTP pools in *S. Typhimurium* (Ginther & Ingraham, 1974; Ingraham & Ginther, 1978). Several lines of evidence in our study unambiguously established the secretory nature of this enzyme in *S. Typhimurium* (Fig. 1 and Supplementary Fig. S2) and thus corroborated reports for other pathogenic microbes (Coutinho-Silva *et al.*, 2001; Gounaris *et al.*, 2001; Punj *et al.*, 2000; Zaborina *et al.*,

Table 1. Kinetic parameters of recombinant NDKs

The NTP-synthesizing assay was carried out as described in Methods and monitored the incubation of purified vNDK (150 ng per reaction) with different concentrations (5–400 μ M) of ADP. K_m and V_{max} values were calculated from Lineweaver–Burk plots, and the results are expressed as mean $\pm$ SD. For calculating k_{cat} values, the molecular mass of recombinant vNDK was taken as 18.05 kDa.

NDK	K_m (μ M)	k_{cat} (s^{-1})	k_{cat}/K_m (μ M $^{-1}$ s^{-1})	Reference or source
sNDK	26.57 ± 1.1	78.03 ± 2.08	2.94 ± 0.02	Dar & Chakraborti (2010)
mNDK	23.7 ± 1.1	287.71 ± 4.2	12.14 ± 6	Tiwari <i>et al.</i> (2004)
vNDK	19.4 ± 2.45	25.3 ± 3.30	1.3 ± 0.5	This study

Fig. 6. Complementation of the Δ sndk strain with NDK from *V. cholerae*. (a) Expression of vNDK in the Δ sndk strain. Uninduced (UI) or induced (I) samples of the Δ sndk strain containing insert (pGEX-Kan-vNDK) or vector (pGEX-Kan). Cell lysates were prepared, resolved on SDS-PAGE and stained with Coomassie brilliant blue (upper panel) or transferred to a nitrocellulose membrane. The blot was probed with anti-mNDK antibody (middle panel) or anti-GST antibody (lower panel). The arrow indicates vNDK. The positions of molecular mass markers are indicated. (b) NTP-synthesizing ability of the complemented strain. Concentrated cell-free supernatant samples of the wild-type (S.t), Δ sndk or vNDK in Δ sndk, or the vector in Δ sndk were incubated with CDP or GDP and then resolved on a TLC plate. The formation of the products was visualized in a phosphorimaging device and by autoradiography. (c) Effect of vndk complementation of the Δ sndk strain on ATP-induced cell death. J774 cells were incubated with buffer, ATP or ATP in the presence of concentrated cell-free culture supernatant from the wild-type or Δ sndk strain harbouring pGEX-Kan-vNDK or pGEX-Kan-sNDK for 4 h, and LDH release was monitored. Δ vndk represents the NDK-deleted strain of *V. cholerae*. Results are mean and SD ($n=3$). The LDH release from cells alone was treated as a blank and was subtracted from each test sample. ***, $P<0.001$. Inset: LDH release from cells in response to no treatment (cells only) or treatment with 10 $\times$ concentrated culture supernatant from *S. Typhimurium* (S.t) or sNDK-complemented Δ sndk (sNDK + Δ sndk) or vNDK-complemented Δ sndk (vNDK + Δ sndk) in the absence of ATP.

counterpart from *V. cholerae* exhibited cytotoxic behaviour (Fig. 4c). Considering that NDKs from *S. Typhimurium* and *V. cholerae* show 65% identity at the amino acid level (Supplementary Fig. S3), that both are secretory (Figs 1 and 4a) and that they are able to utilize ATP as substrate (Fig. 5a, b), their bifunctional nature (Chopra *et al.*, 2003; Punj *et al.*, 2000; Yilmaz *et al.*, 2008) is quite puzzling.

To resolve this issue, we exploited a Δ sndk strain, in which other ATP-utilizing enzymes secreted by *S. Typhimurium* are unable to carry out the function of NDK and are unable to modulate the ATP-induced cytotoxicity of J774 cells (Fig. 3b). Since the biochemical characteristics of NDKs from intra- and extracellular pathogens are similar, we wanted to determine whether the deficiencies of the Δ sndk strain could be functionally complemented by vNDK. To our surprise, we found that the concentrated culture supernatant of the vNDK-transformed Δ sndk strain could synthesize NTPs and was also able to prevent the ATP-induced cytotoxicity of J774 cells, as observed for secretory sNDK (Fig. 6b, c). To address the discrepancy in the behaviour of secretory vNDK from wild-type *V. cholerae* (Fig. 4b) and from the vNDK-transformed Δ sndk strain with respect to the prevention/modulation of ATP-induced J774 macrophage cell lysis (Fig. 6c), we further generated an ndk-deleted strain of *V. cholerae* (Δ vndk). The concentrated culture supernatant from this strain, when incubated with J774 cells, exhibited the same level of cell killing as that observed in the presence of ATP alone (Fig. 6c), suggesting that secretory NDK from an extra-

2000). We further ascertained that the concentrated culture supernatant of this intracellular pathogen could prevent ATP-induced LDH release (Fig. 2b), while its extracellular

cellular pathogen per se does not contribute to the cytotoxicity of mouse macrophage cells. Thus our findings address a long-standing unresolved issue concerning the pathogen-specific bifunctional behaviour of NDK, and certainly demand a revisit of the traditional concept.

NDKs from intra- and extracellular pathogens might have different roles to play, despite being biochemically indistinguishable. This is very likely because of their distinct physiological niches. In fact, pathogens are known to secrete different proteins, factors or even effectors, which either regulate different processes or modulate the function of a known protein(s), and thus may render toxicity to the host cells for their survival (Paumet *et al.*, 2009; Garcia-del Portillo & Finlay, 1995; Prost *et al.*, 2007). Further study in this direction is necessary to illuminate such a perplexing issue. Nonetheless, our results for what we believe to be the first time lend credence to the fact that the intrinsic properties of NDKs from intra- and extracellular pathogens cannot be distinguished.

ACKNOWLEDGEMENTS

We are indebted to Dr A. Mukhopadhyay, National Institute of Immunology (NII), New Delhi, India, for providing us with an *S. Typhimurium* strain. We also gratefully acknowledge the kind receipt of pGP704 and *E. coli* strain SM102pir from Dr S. Ray Chowdhury, Institute of Microbial Technology (IMTECH), Chandigarh, India. We are thankful to Dr Alok Mondal for critical reading of the manuscript and to Mr J. Prasad for providing us with excellent technical assistance. Senior Research Fellowships (H. H. D. and D. P.) from the Council of Scientific and Industrial Research (CSIR), New Delhi, India, and an in-house grant from IMTECH, Chandigarh, India, are gratefully acknowledged.

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Edited by: W. Bitter