An additional mechanism of ribosome-inactivating protein cytotoxicity: degradation of extrachromosomal DNA

Emmanuelle NICOLAS, Ian D. GOODYER and Theodore F. TARASCHI[1]

Department of Pathology, Anatomy and Cell Biology, Jefferson Medical College, 1020 Locust St., Philadelphia, PA 19107, U.S.A.

Inhibition of protein synthesis by cleavage of the N-glycosidic bond of a specific adenine of 28 S rRNA has been accepted as the mechanism by which plant ribosome-inactivating proteins (RIPs) cause cytotoxicity. The cytotoxic action of gelonin on *Plasmodium falciparum* malaria parasites appears to occur by a different mechanism. Parasite intoxication, which is manifested by mitochondrial dysfunction and lack of nucleic acid synthesis in the erythrocytic cycle following exposure to the toxin, is caused by the elimination of the parasite 6 kb extrachromosomal (mitochondrial) DNA. This is the first report which demonstrates that the DNA-damaging activities of RIPs observed *in vitro* can contribute to their cytotoxicity.

INTRODUCTION

A pressing need exists to develop new antimalarial agents, due to the rapid development of drug resistance [1]. Historically, asexual blood-stage parasites were considered to be isolated in a safe haven provided by the surrounding erythrocyte. Consequently, the search for chemotherapeutic agents focused on small membrane-permeable drugs. We have described the existence of a pathway wherein plasma (macro)molecules bypass the erythrocyte membrane and are internalized into intra-erythrocytic parasites by fluid-phase endocytosis [2]. This discovery led us to propose a new concept in antimalarial drug development: the parasite no longer should be considered protected from external toxic macromolecules due to an intracellular location [2].

To test this hypothesis, we selected the ribosome-inactivating proteins (RIPs), which are well known for their ability to inhibit protein synthesis by removing a specific adenine of the 28 S rRNA [3,4]. Plant RIPs are categorized into two groups: type-I RIPs, such as gelonin, bryodin or trichosanthin, are single-chain proteins, and type-II RIPs, such as ricin or abrin, have an additional chain [3]. Type II RIPs are among the most toxic substances known because the additional chain has a lectin property that allows cellular internalization and access to the intracellular target. Investigations of the cytotoxicity of the type-II RIPs on asexual malaria parasites are precluded by their haemagglutination activity.

In this investigation, we report that gelonin is cytotoxic to *Plasmodium falciparum*-infected erythrocytes. The cytotoxicity is not due to the classical mechanism of inhibition of protein synthesis. Parasite killing is found to be associated with the loss of the parasite mitochondrial DNA. This observation is discussed in the light of reports of RIPs damaging DNA in cell-free assays [5–16].

MATERIALS AND METHODS

Parasites

Clone 5 of the FCR Gambian stain of *P. falciparum* was grown and synchronized within 4 h as described previously [17].

RIP

Lyophilized gelonin (lot 121H4035) was from Sigma (St. Louis, MO, U.S.A.). Gelonin was resuspended in culture medium and sterile filtered with a 0.22 μm cellulose acetate filter (Costar, Cambridge, MA, U.S.A.) before use. The protein concentration was determined from its absorbance spectrum using A of a 1 % solution at 280 nm = 6.7 [18].

Protein and nucleic acids syntheses

Malaria-infected erythrocytes [10^8 cells; 3 % haematocrit/4 % parasitaemia in 100 μl of culture medium] were treated as indicated in the Figure captions and exposed to [4,5-^{3}H]isoleucine (102 Ci/mmol) or [8-^{3}H]hypoxanthine (4.7 Ci/mmol) (Amersham, Arlington Heights, IL, U.S.A.) at the indicated times. Protein and nucleic acid syntheses were quantified by measurement of incorporation of radioactivity into trichloroacetic acid-precipitable material. The incorporation of radioactive hypoxanthine into RNA and DNA was determined as described by de Rojas and Wasserman [19].

DNA analysis

To minimize mechanical or enzymic damage to the DNA during sample processing, cells were embedded directly into agarose plugs and deproteinized in the presence of a denaturing concentration of detergent and EDTA [20]. Each plug was prepared from 1 ml of infected red blood cells (IRBC) suspension (10^9 cells; 3 % haematocrit/4 % parasitaemia) treated as indicated in the Figure captions. Deproteinized plugs were digested with *Hin*dIII (Promega, Madison, WI, U.S.A.). DNA digests were separated by conventional agarose electrophoresis [1 % (w/v) Seakem Gold, FMC], with λDNA/*Hin*dIII fragments and a 1 kb DNA ladder (Gibco–BRL, Gaithersburg, MD, U.S.A.) as size standards, and transferred on to Zeta Probe GT nylon membranes (Bio-Rad, Hercules, CA, U.S.A.). Southern-blot analysis was carried out by the standard protocol of the manufacturer, except that 5 % (w/v) SDS was used and the hybridization temperature was 50 °C for the organellar DNAs. The probes used were the 1616 bp *Bam*HI/*Pst*I (Promega) fragment of the *Plasmodium* FCR3 HRP1 coding sequence [21], the 6 kb element

Abbreviations used: RIP, ribosome-inactivating protein; IRBC, infected red blood cells.

[1] To whom correspondence should be addressed.

or a 700 bp segment that spans part of the small subunit rRNA gene of the 35 kb DNA [22].

Mitochondrial function

Cells were incubated with 0.5 μM mitochondrial membrane potential dye, tetramethylrhodamine ethyl ester (Molecular Probes, Eugene, OR, U.S.A.), for 30 min. The transmitted light and fluorescence images were collected with a Bio-Rad MRC 600 confocal fluorescence microscope system, as described previously [23].

RESULTS

Treatment of IRBC with gelonin inhibits parasite protein and nucleic acid syntheses

Various concentrations (10^{-6}–10^{-7} M) of gelonin were added to IRBC at the ring stage [8($\pm$2) h post-invasion]. Cytotoxicity was assayed in trophozoite-stage infections (29–35 h post-invasion) in the first and second erythrocytic cycles by measuring the incorporation of [^{3}H]isoleucine and [^{3}H]hypoxanthine into parasite protein and nucleic acid respectively, and comparing the values with those obtained for untreated IRBC. Gelonin, at these concentrations, did not produce cytotoxicity in the first erythrocytic cycle (results not shown). However, when these activities were measured in the subsequent erythrocytic cycle (Figure 1), protein synthesis was reduced to 53 % in IRBC incubated with 10^{-6} M gelonin, and nucleic acid synthesis was even more strongly affected in these IRBC, being reduced to 17 % of the control value. Since the cells had been washed and resuspended in toxin-free medium at the schizont stage (40 h post-invasion) of the first erythrocytic cycle, and the infections were highly synchronous, the inhibitory effects were not due to an internalization of the protein during parasite invasion.

Examination of Giemsa-stained blood smears at the ring stage in the second erythrocytic cycle revealed that the low incorporation of radioactive precursors in gelonin-treated cells was not due to reduced parasite invasiveness, as the parasitaemia of control and treated cultures was not significantly different (results not shown). Blood smears made later in the second erythrocytic cycle (at 25 and 40 h post-invasion) revealed that the treated (10^{-6} M gelonin) parasites failed to mature properly and eventually died, resulting in the virtual elimination of parasitaemia.

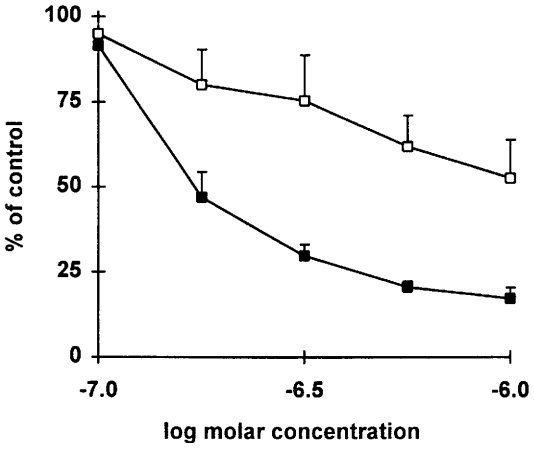

Figure 1 *P. falciparum* protein and nucleic acid syntheses at the trophozoite stage of the erythrocytic cycle following exposure to gelonin

IRBC were exposed to various concentrations of gelonin at 8($\pm$2) h post-invasion. At 40 h post-invasion, cells were washed and returned to culture in toxin-free medium after a reduction of the parasitaemia by the addition of uninfected erythrocytes. Radioactive isoleucine (□) or hypoxanthine (■) was added at 29 h post-invasion in the second cycle. Cells were harvested 4 h later for quantification of incorporation. Data are percentages of incorporation of radioactive label into toxin-treated IRBC relative to untreated IRBC. Untreated cells gave 2967 c.p.m. for isoleucine and 34114 c.p.m. for hypoxanthine.

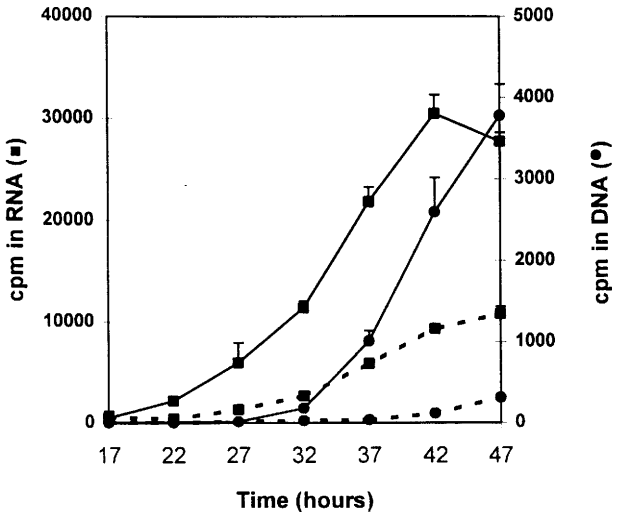

Figure 2 RNA and DNA syntheses during the erythrocytic cycle following exposure to gelonin

Radioactive hypoxanthine was added at 12 h post-invasion in the second cycle, to untreated IRBC (solid lines) or to IRBC treated with 10^{-6} M gelonin (broken lines) as described in Figure 1. Incorporation into RNA (cpm in RNA; squares) and DNA (cpm in DNA; circles) was quantified at the indicated time points.

To further characterize the nucleic acid synthesis inhibition, the incorporation of [^{3}H]hypoxanthine into RNA and DNA in gelonin-treated cells (10^{-6} M) or untreated cells was performed at different stages of the erythrocytic cycle and measured (Figure 2). In treated IRBC, RNA synthesis was reduced to 20–30 % of control values between 22 and 42 h post-invasion, whereas DNA synthesis, which commences at approx. 34 h in untreated cells [24,25], was virtually abolished.

Ribosome-inactivating property of gelonin does not contribute to cytotoxicity

The antiviral activity of the RIP pokeweed antiviral protein was maintained after treatment of the protein for 5–10 min at 100 °C [26]. Under these conditions, gelonin loses its ribosome-inactivating property [18]. When IRBC were treated with 10^{-6} M boiled gelonin, as described in Figure 1, the incorporation of [^{3}H]isoleucine and [^{3}H]hypoxanthine into parasite proteins and nucleic acids was found to be 53 % and 19 % of control values, respectively. These results, which are similar to those obtained for IRBC incubated with untreated gelonin (Figure 1), appear to exclude ribosome inactivation as the origin of cytotoxicity.

Treatment of IRBC with gelonin causes the elimination of the parasite extrachromosomal 6 kb DNA

RIPs have been shown to damage DNA *in vitro* [5–16]. Boiling removes the ribosome-inactivating property of ricin A-chain without substantially diminishing its ability to damage supercoiled DNA [8]. This observation, in combination with the results in Figures 1 and 2, raised the possibility that gelonin

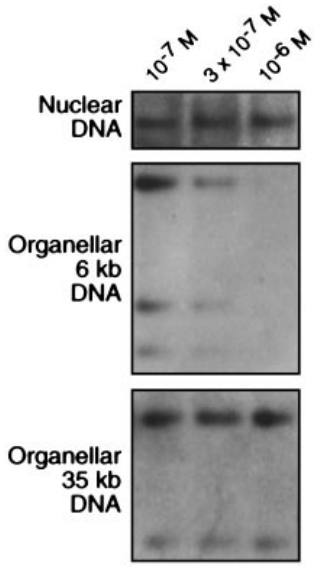

Figure 3 Southern-blot analysis of *Hin*dIII-digested DNA at the ring stage of the cycle following exposure to gelonin

Cells were treated as in Figure 1 with the indicated concentrations of gelonin, and embedded in agarose when parasites were in the ring stage of the second erythrocytic cycle. Plugs were processed for Southern-blot analysis with probes for nuclear DNA (HRP1) and 6 kb and 35 kb organellar DNAs. The HRP1 band is at approx. 10 kb. The bands for digested organellar DNAs are at 1.2, 1.6 and 3.1 kb for 6 kb DNA and 1.9 and 0.8 kb for 35 kb DNA, in agreement with the *Hin*dIII restriction maps [21,40].

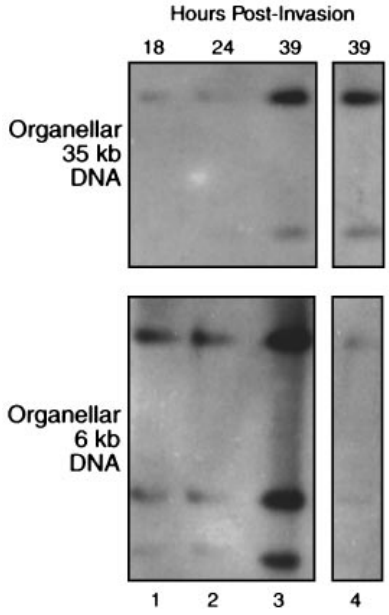

Figure 4 Southern-blot analysis of *Hin*dIII-digested DNA during the cycle of exposure to gelonin

IRBC, untreated (lanes 1–3) or treated with 10^{-6} M gelonin (lane 4), were embedded in agarose at the indicated times. Plugs were analysed as in Figure 3 for the content of organellar DNAs.

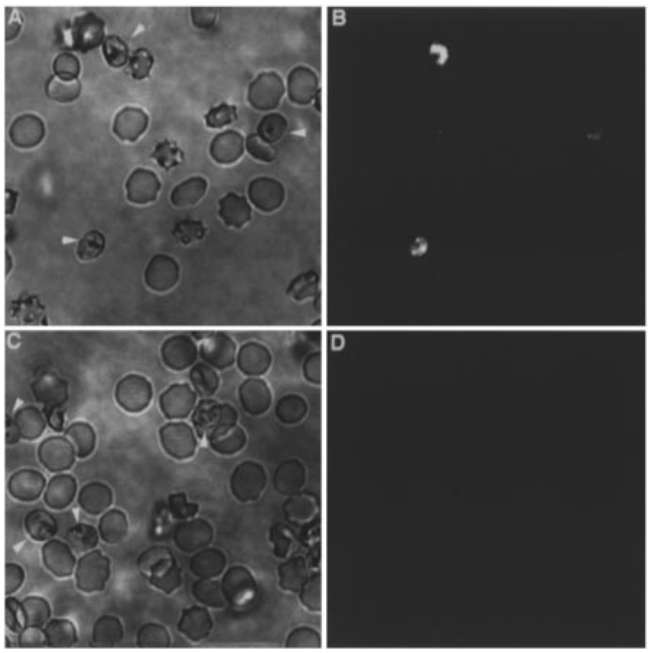

Figure 5 Loss of mitochondrial function in gelonin-treated parasites

Confocal fluorescence images of IRBC treated as in Figure 3 and incubated with the fluorescent dye tetramethylrhodamine ethyl ester. Parasitized erythrocytes are indicated by the white arrowheads in the transmitted images. (**A**) and (**B**) Untreated IRBC; (**C**) and (**D**) gelonin-treated IRBC; (**A**) and (**C**) transmitted light; (**B**) and (**D**) fluorescence images.

produced cytotoxicity through an action against parasite DNA. In addition to nuclear DNA, *Plasmodium* has two extrachromosomal DNAs with organelle-like characteristics, referred to as 6 kb and 35 kb DNA [24]. Southern-blot analysis of *Hin*dIII-digested DNA from IRBC removed from culture at the ring stage of the second erythrocytic cycle revealed that gelonin caused a dose-dependent decrease in the amount of 6 kb DNA, whereas the amounts of 35 kb DNA and nuclear DNA (measured by probing for DNA encoding the parasite protein HRP1) were unaltered (Figure 3). Boiled gelonin had similar effects to untreated gelonin (results not shown).

To determine whether the loss of the 6 kb DNA observed in the second erythrocytic cycle was due to its degradation, or inhibition of its replication during the first cycle, or both, the amount of 6 kb DNA in gelonin-treated cells after organellar DNA replication was compared with the amount of 6 kb DNA in untreated cells before and after organellar DNA replication [25]. Figure 4 (lanes 1–3) showed the expected evolution of the 6 kb DNA in control cells, with similar amounts detected before replication (18 and 24 h post-invasion), and a large increase after replication (39 h post-invasion). Lane 4 in Figure 4 indicated that at 39 h, gelonin-treated cells contained less 6 kb DNA than the control cells before replication. Similar amounts of 35 kb DNA in treated and untreated cells (lanes 3 and 4) verified that this result was not an artifact of sample loading. Thus the loss of 6 kb DNA observed in the second cycle was due to the degradation (and inhibition of replication) of the DNA during the cycle of exposure to the toxin.

Treatment of IRBC with gelonin causes mitochondrial dysfunction

The 6 kb element has been found to encode for three components of an electron-transport system (apocytochrome *b*, subunits I and III of cytochrome *c* oxidase) normally encoded by the mitochondrial genome and is therefore referred to as the mitochondrial DNA [24]. As the 6 kb transcripts are most abundant

in late trophozoite- and schizont-stage infections [27], one can predict that the absence of the 6 kb DNA may cause mitochondrial dysfunction. Membrane-potential-sensitive dyes have been used to probe the functional status of mitochondria in *Plasmodium* [28,29]. When incubated with the membrane-potential-sensitive dye tetramethylrhodamine ethyl ester, the mitochondria in $> 99\,\%$ of the untreated IRBC were labelled (Figure 5). By contrast, $95\,\%$ of trophozoite-stage parasites (approx. 30 h post-invasion), treated in the previous erythrocytic cycle with gelonin (10^{-6} M), failed to accumulate the dye, indicating that they did not maintain a membrane potential.

DISCUSSION

RIPs, free or conjugated to ligands, are currently being evaluated for their anti-cancer and anti-HIV efficacy in humans [30–32]. Anti-viral uses in agribiology have been proposed [33]. Some reports suggested that cytotoxicity may not only be due to inactivation of cellular protein synthesis [9,10,34–39]. Although our study was undertaken to examine the feasibility of using macromolecules as chemotherapeutic agents against malaria, which was validated as a viable approach, it also provided direct evidence for an additional mechanism of RIP cytotoxicity. In this system, the cytotoxicity (which was delayed to the erythrocytic cycle following the exposure to the toxin and manifested by the loss of parasite mitochondrial function and inhibition of nucleic acid synthesis) was found to be associated with the loss of the parasite extrachromosomal 6 kb DNA.

It was not surprising that this damage was lethal to the parasites, considering the high conservation of the nucleotide sequences of the 6 kb mitochondrial element between different *Plasmodium* species [40] and the observations that functional mitochondria are critical for the growth of erythrocytic-stage malaria parasites [24,41]. The inhibition of the dihydro-orotate dehydrogenase by atovaquone [42], which acts on complex III of the mitochondrial respiratory chain [43], indicates that mitochondria are involved in the *de novo* biosynthesis of pyrimidines. This explains why the cytotoxicity of gelonin was manifested as an inhibition of nucleic acid synthesis.

Although the cytotoxicity of RIPs has been thought to be due to the inhibition of protein synthesis, we describe, for the first time, a cytotoxic effect associated with the loss of DNA. Our attempt to confirm that the cytotoxicity was due to gelonin rather than a contaminant, by using a bacterial recombinant gelonin, was unsuccessful, since it was non-toxic to the parasites. Failure in using this approach has been reported previously. It could indicate the importance of glycosylation [44] or other, yet unidentified, differences between the native plant and the recombinant bacterial proteins important for their activity *in vivo* [45].

Plant RIPs have been described to have an additional DNase activity [5–8,10–12,14,16]. The potential contribution of this activity to their cytotoxicity has not been given serious consideration due to concerns about possible contamination. By using a different approach, Stirpe and co-workers have described the catalytic removal of multiple adenines from various substrates, including DNA [9,13,15]. They have demonstrated that this property is common to all the 52 RIPs tested and ruled out the possibility that this activity could be due to a contaminant [15]. Recent studies have shown that single-stranded DNA is the preferred substrate for the nuclease activity [14,16]. Gelonin was identified as the polypeptide responsible for the degradation of single-stranded DNA present in preparations of plant and *Escherichia coli* recombinant gelonin by zymography [16]. Based on the ability of the DNA glycosylases/abasic site (AP) lyases to remove bases and induce strand scission [46], and the instability of DNA containing apurinic sites [47], it is likely that the removal of adenines from DNA and the strand scissions are connected. Further studies will determine whether the strand breakage *in vitro* at abasic sites produced by RIPs occurs spontaneously, as suggested in [15], or is the consequence of a RIP lyase activity. The breakage at the RIP-induced abasic sites *in vivo* could be performed by cellular enzymes. If RIPs also have an AP lyase activity, they could also degrade abasic sites in DNA that occur spontaneously or are created by other cellular enzymes. It is possible that in the malaria parasites treated with gelonin, abasic sites in the 6 kb DNA are further damaged by parasite enzymes, producing the observed loss of the 6 kb DNA. In the light of the activities reported for the RIPs *in vitro*, it is reasonable to suggest that the damage to the 6 kb DNA is initiated directly by gelonin. The extent of the damage attributable to gelonin (removal of adenines, degradation or both) remains to be determined.

The 35 kb chloroplast-like malaria DNA was not affected by gelonin treatment of IRBC, suggesting that factors other than an extrachromosomal location are likely to influence the susceptibility to DNA damage and its consequences. In mammalian cells treated with RIPs, effects on mitochondrial DNA have not been sought. The susceptibility of the mitochondrial DNA to gelonin could be peculiar to *Plasmodium*, which has mitochondria that are genetically, structurally and functionally distinct from those of mammalian cells [24,48]. Replication of the 6 kb DNA involves multiple recombinations between linear, tandem arrays, in a manner similar to the recombination-dependent replication process used by phage T4 [48]. During replication, the 3′-overhanging ends of incompletely replicated linear duplexes invade homologous regions of other linear or circular duplexes, causing branch migration and generation of uni- or bi-directional replication forks, single-stranded DNA molecules and rolling circles. Single-stranded DNA containing abasic sites is more likely to break than double-stranded DNA [49]. This may explain the apparent selectivity of damage on the 6 kb DNA. Interestingly, the selective killing of HIV-infected cells by onconase, an amphibian ribonuclease, was due to an unexpected selectivity for viral nucleic acid species [50]. Whereas *Plasmodium* 6 kb DNA is the gelonin target in parasitized erythrocytes, other DNAs may be vulnerable. The information gleaned from the malaria system may be useful for identifying other parasites or infectious organisms with similar biology that may be susceptible to the DNA-damaging action of RIPs.

We thank Akhil Vaidya for the gift of the probes for the organellar DNAs and for helpful discussions. Supported by USPHS grant AI27247 and Agency for International Development grant 936-6001.27 to T. F. T.

REFERENCES

1 White, N. J. and Norsten, F. (1993) Curr. Opin. Infect. Dis. **6**, 323–330
2 Pouvelle, B., Spiegel, R., Hsiao, L., Howard, R. J., Morris, R. L., Thomas, A. P. and Taraschi, T. F. (1991) Nature (London) **353**, 73–75
3 Barbieri, L., Battelli, M. G. and Stirpe, F. (1993) Biochim. Biophys. Acta **1154**, 237–282
4 Endo, Y., Mitsui, K., Motizuki, M. and Tsurugi, K. (1987) J. Biol. Chem. **262**, 5908–5912
5 Li, M.-X., Yeung, H.-W., Pan, L.-P. and Chan, S. I. (1991) Nucleic Acids Res. **19**, 6309–6312
6 Huang, P. L., Chen, H.-C., Kung, H.-f., Huang, P. L., Huang, P., Huang, H. I. and Lee-Huang, S. (1992) BioFactors **4**, 37–41
7 Go, T. T. M., Yeung, H. W. and Fong, W. P. (1992) Life Sci. **51**, 1347–1353
8 Ling, J., Liu, W.-y. and Wang, T. P. (1994) FEBS Lett. **345**, 143–146
9 Barbieri, L., Gorini, P., Valbonesi, P., Castiglioni, P. and Stirpe, F. (1994) Nature (London) **372**, 624
10 Lee-Huang, S., Huang, P. L., Chen, H.-C., Huang, P. L., Bourinbaiar, A., Huang, H. I. and Kung, H.-F. (1995) Gene **161**, 151–156

11 Ling, J., Li, X.-d., Wu, X.-h. and Liu, W.-y. (1995) Biol. Chem. Hoppe-Seyler **376**, 637–641
12 Ling, J., Liu, W.-y. and Wang, T. P. (1995) Biochim. Biophys. Acta **1252**, 15–22
13 Barbieri, L., Valbonesi, P., Gorini, P., Pession, A. and Stirpe, F. (1996) Biochem. J. **319**, 507–513
14 Roncuzzi, L. and Gasperi-Campani, A. (1996) FEBS Lett. **392**, 16–20
15 Barbieri, L., Valbonesi, P., Bonora, E., Gorini, P., Bolognesi , A. and Stirpe, F. (1997) Nucleic Acids Res. **25**, 518–522
16 Nicolas, E., Beggs, J. M., Haltiwanger, B. M. and Taraschi, T. F. (1997) FEBS Lett. **406**, 162–164
17 Pouvelle, B., Farley, P. J., Long, C. A. and Taraschi, T. F. (1994) J. Clin. Invest. **94**, 413–417
18 Stirpe, F., Olsnes, S. and Pihl, A. (1980) J. Biol. Chem. **255**, 6947–6953
19 de Rojas, M. O. and Wasserman, M. (1985) Trans. R. Soc. Trop. Med. Hyg. **79**, 792–796
20 Walker, P. R., Kokileva, L., LeBlanc, J. and Sikorska, M. (1993) BioTechniques **15**, 1032–1040
21 Pologe, L. G., Pavlovec, A., Shio, H. and Ravetch, J. V. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 7139–7143
22 Gardner, M. J., Feagin, J. E., Moore, D. J., Spencer, D. F., Gray, M. W., Williamson, D. H. and Wilson, R. J. M. (1991) Mol. Biol. Parasitol. **48**, 77–88
23 Gormley, J. A., Howard, R. J. and Taraschi, T. F. (1992) J. Cell Biol. **119**, 1481–1495
24 Feagin, J. E. (1994) Annu. Rev. Microbiol. **48**, 81–104
25 Smeijsters, L. J. J. W., Zijstra, N. M., de Vries, E., Franssen, F. F. J., Janse, C. J. and Overdulve, J. P. (1994) Mol. Biol. Parasitol. **67**, 115–124
26 Fukaya, N. and Taniguchi, T. (1979) Phytopathol. Z. **94**, 132–138
27 Feagin, J. E. and Drew, M. E. (1995) Exp. Parasitol. **80**, 430–440
28 Divo, A. A., Geary, T. G., Jensen, J. B. and Ginsburg, H. (1985) J. Protozool. **32**, 442–446
29 Kiatfuengfoo, R., Suthiphongchai, T., Prapunwattana, P. and Yuthavong, Y. (1989) Mol. Biochem. Parasitol. **34**, 109–116
30 Kahn, J. O., Gorelick, K. J., Gatti, G., Arri, C. J., Lifson, J. D., Gambertoglio, J. G., Bostrom, A. and Williams, R. (1994) Antimicrob. Agents Chemother. **38**, 260–267
31 Vitetta, E. S., Thorpe, P. E. and Uhr, J. W. (1993) Immunol. Today **14**, 252–259
32 Brinkmann, U. and Pastan, I. (1994) Biochim. Biophys. Acta **1198**, 27–45
33 Shah, D. M., Rommens, C. M. T. and Beachy, R. N. (1995) Trends Biotechnol. **13**, 362–369
34 Teltow, G. J., Irvin, J. D. and Aron, G. M. (1983) Antimicrob. Agents Chemother. **23**, 390–396
35 McGrath, M. S., Hwang, K. M., Caldwell, S. E., Gaston, I., Luk, K.-C., Wu, P., Ng, V. L., Crowe, S., Daniels, J., Marsh, J., Deinhart, T., Lekas, P. V., Vennari, J. C., Yeung, H.-W. and Lifson, J. D. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 2844–2848
36 Olson, M. C., Ramakrishnan, S. and Anand, R. (1991) AIDS Res. Hum. Retroviruses **7**, 1025–1031
37 Lee-Huang, S., Kung, H.-F., Huang, P. L., Bourinbaiar, A. S., Morell, J. L., Brown, J. H., Huang, P. L., Tsai, W.-P., Chen, A. Y., Huang, H. I. and Chen, H.-C. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 12208–12212
38 Rybak, S. M., Lin, J.-J., Newton, D. L., Kung, H.-F., Monks, A., Chen, H.-C., Huang, P. L. and Lee-Huang, S. (1994) Int. J. Oncol. **5**, 88–94
39 Lee-Huang, S., Huang, P. L., Huang, P. L., Bourinbaiar, A. S., Chen, H.-C. and Kung, H. F. (1995) Proc. Natl. Acad. Sci. U.S.A. **92**, 8818–8822
40 Suplick, K., Morrissey, J. and Vaidya, A. B. (1990) Mol. Cell. Biol. **10**, 6381–6388
41 Vaidya, A. B., Lashgari, M. S., Pologe, L. G. and Morrisey, J. (1993) Mol. Biochem. Parasitol. **58**, 33–42
42 Ittarat, I., Asawamahasakda, W. and Meshnick, S. R. (1994) Exp. Parasitol. **79**, 50–56
43 Fry, M. and Pudney, M. (1992) Biochem. Pharmacol. **43**, 1545–1553
44 Johnson, V. G. and Youle, R. J. (1991) Intracellular routing and membrane translocation of diphtheria toxin and ricin, in Intracellular Trafficking of Proteins, pp. 183–225, Cambridge University Press, Cambridge, U.K.
45 Boix, E., Wu, Y., Vasandani, V. M., Saxena, S. K., Ardelt, W., Ladner, J. and Youle, R. J. (1996) J. Mol. Biol. **257**, 992–1007
46 Sun, B., Latham, K. A., Dodson, M. L. and Lloyd, R. S. (1995) J. Biol. Chem. **270**, 19501–19509
47 Lindhal, T. and Andersson, A. (1972) Biochemistry **11**, 3618–3623
48 Preiser, P. R., Wilson, R. J., Moore, P. W., McCready, S., Hajibagheri, M. A., Blight, K. J., Strath, M. and Williamson, D. H. (1996) EMBO J. **15**, 684–693
49 Lafleur, M. V. M., Woldhuis, J. and Loman, H. (1981) Nucleic Acids Res. **9**, 6591–6599
50 Saxena, S. K., Gravell, M., Wu, Y. N., Mikulski, S. M., Shogen, K., Ardelt, W. and Youle, R. J. (1996) J. Biol. Chem. **271**, 20783–20788

Received 20 January 1997/21 May 1997; accepted 3 June 1997