Biochemical and Biophysical Research Communications **260,** 105–110 (1999)
Article ID bbrc.1999.0712, available online at http://www.idealibrary.com on IDEAL®

Induction of Cell Proliferation and Apoptosis: Dependence on the Dose of the Inducer

Tanya Das,* Gaurisankar Sa,† Pratima Sinha,* and Prasanta K. Ray*,[1]
**Immunotechnology Section and †Animal Physiology Section, Bose Institute, Calcutta, India*

Received March 22, 1999

Protein A (PA) of *Staphylococcus aureus* is known as an immunomodulator. In a search of the molecular mechanism(s) of PA-induced immunocyte potentiation, we found dose-dependent binding of PA (0.01 to 100 μg/ml PA) to the mice splenic lymphocytes. Interestingly, treatment of 1 μg PA/20 g mice increased the splenic lymphocyte number ~5-fold over control but at a 10-μg dose the cell number was decreased compared with a 1-μg dose. Flow cytometric analysis of cell-cycle phase distribution of nuclear DNA in splenic lymphocytes showed that at a 1-μg dose, PA shifted the cell-cycle phases from G0/G1 to S and G2/M supporting the pro-proliferative role of PA. In contrast, the same inducer increased the sub-G1 cell population at a 10-μg dose indicating the breakdown of cellular DNA. These findings were supported by DNA ladder formation and nuclear breakdown at this higher dose. Further studies revealed that at a 1-μg dose, the level of the pro-proliferative/anti-apoptotic protein bcl-2 was increased in splenic lymphocytes whereas at a 10-μg dose it showed a decreasing trend. In contrast, concentrations of proapoptotic proteins, p53 and bax, were increased at a 10-μg dose. A search of the mechanism(s) of such differential action of PA at these two doses revealed that the lower dose of PA upregulated the production of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) to the extent which has already been reported by our laboratory to be beneficial to the host. However, at a larger dose, much higher release of TNF-α and interleukin-2 (IL-2) may account for the apoptosis of splenic cells. All these findings indicated that the cross-talk between all these pro- and anti-apoptotic factors may contribute to maintain a balance between growth and death of cells and may be one of the important factors deciding whether a cell would follow a proliferative pathway or an apoptotic pathway.

Disease and drug related immunodeficiency is one of the major concerns of the scientists all over the world. Many of the deadly diseases like cancer, AIDS etc. as well as most of the drugs used in the treatment of these diseases cause severe immunodeficiency (1, 2). As a result, patient often dies out of secondary infections than by the disease itself. Thus, recent trends are the usage of immunomodulators, e.g., interleukins (ILs), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) (3–5) and many others, in such situations to overcome the immunosuppression.

Protein A (PA) of *Staphylococcus aureus* displays diverse immunostimulatory and biological response modifying properties (6–12). It can activate B lymphocytes by binding to the V_H sequence of the B cell receptor (6) and stimulate T cells by cross linking with the MHC II molecules on the T cell surface (7). As a consequence, various immune responses are potentiated, e.g., complement activity (8), phagocytosis in macrophages (9), generation of LAK cells (10), NK cell activity (11) etc. Recent studies from our laboratory demonstrated that PA induces the production of different cytokines (12, 13), increases nitric oxide production (14), increases $CD4^+$ and $CD8^+$ T lymphocytes number (15), and initiates a signaling pathway in splenic lymphocytes (14, 16). These multifarious properties of PA have made it suitable for being used as an effective immunomodulator which can restore the depressed immune function. In fact, PA has already been found to regress tumors and increase the percent survival of tumor bearing mice (17, 18). Furthermore, PA has been found to ameliorate the toxicity-related immunodepression in mice treated with immunosuppressive drugs such as azidothymidine, cyclophosphamide etc. (19, 20).

For the past few years we are engaged in studying the mechanism of immunorestoration in immunodefi-

[1] To whom correspondence should be addressed at Bose Institute, P-1/12 CIT Scheme VII M, Calcutta 700 054, India. Fax: 91-33-3343886. E-mail: pkray@boseinst.ernet.in.

Abbreviations used: CD, cluster of differentiation; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; IL, interleukin; IFN-α, interferon-α; IFN-γ, interferon-γ; LAK, lymphokine-activated killer cell; NK, natural killer; PA, protein A; PI, propidium iodide; TNF-α, tumor necrosis factor-α.

0006-291X/99 $30.00

cient host using PA. In the present study, we have observed that the effects of PA in splenic lymphocytes are highly dose dependent. At one dose, it is mitogenic and at a higher dose it is proapoptotic. Further studies revealed that such differential effects of this bacterial protein is highly controlled by the cross-talk between different Th1 cytokines, possibly triggering pro- and anti-apoptotic gene expressions in splenic lymphocytes.

MATERIALS AND METHODS

Animals. Male Swiss albino mice ~20 g body wt were acclimatized for a week before starting the experiment. They were maintained on a commercial pellet diet and fed water *ad libitum*.

Reagents. Protein A and FITC-PA were purchased from Pharmacia Fine Chemicals, Sweden. RPMI 1640 and fetal bovine serum were obtained from Gibco BRL, U.S.A. Cycle TEST PLUS DNA reagent kit was procured from Becton–Dickinson Immunocytometry system, U.S.A., and Apoptotic DNA Ladder Kit was obtained from Boehringer-Mannheim, U.S.A. Polyclonal anti-bax antibody, FITC-conjugated goat anti-mouse and goat anti-rabbit antibodies were procured from Pharmingen, U.S.A. Polyclonal anti-bcl-2 antibody was purchased from Santa-Cruz, U.S.A., and monoclonal anti-p53 antibody was a kind gift from Prof. G. C. Das, U.S.A. Cytokine kits were purchased from Genzyme, U.S.A. All other chemicals were procured from Sigma Chemical Co., U.S.A.

Flow cytometric analysis of protein A binding. PA binding assays on mice splenic lymphocytes (1×10^6 cells) were performed at 37°C for 30 min. Various concentrations (0.01–100 μg/ml) of FITC-PA were used for this assay in PBS, pH 7.5. Nonspecific binding was estimated in the presence of FITC alone. Cells were washed thoroughly and analyzed on a flow cytometer equipped with 488-nm argon laser light source and a 515-nm band pass filter for FITC-fluorescence (FL1-H). Total 10,000 events were acquired for analysis using CellQuest software. Simultest LeucoGATE was used to reduce debris, monocytes, granulocytes or other contamination, if any, and dot plot analysis of FL1-H (*x*-axis; FITC-fluorescence) versus FL2-H (*y*-axis) has been shown in logarithmic fluorescence intensity.

Flow cytometric analysis of cell-cycle phase distribution of nuclear DNA. For the determination of cell-cycle phase distribution of nuclear DNA, splenic lymphocytes (1×10^6 cells) harvested from normal or PA-treated (1 μg or 10 μg PA/20g body wt biweekly for two weeks) Swiss albino mice were fixed with 3% *p*-formaldehyde for 30 min, trypsinized, and nuclear DNA was labeled with propidium iodide (PI, 125 μg/ml) after RNase treatment using Cycle TEST PLUS DNA reagent kit. Cell-cycle phase distribution of nuclear DNA was determined on FACS Calibur using CellQuest software (Becton–Dickinson), fluorescence (FL2-A) detector equipped with 488-nm argon laser light source and 623-nm band pass filter (linear scale, Becton–Dickinson). Total 10,000 events were acquired for analysis. Cells were properly gated as described earlier and analysis of flow cytometric data was performed using ModFit software (Becton–Dickinson). Histogram display of FL2-A (*x*-axis, PI-fluorescence) versus counts (*y*-axis) has been displayed.

Oligonucleosomal fragmentation of nuclear DNA. Genomic DNA from 1×10^6 splenic lymphocytes of mice (treated with or without PA as mentioned above) were isolated using Apoptotic DNA Ladder Kit and separated in 2% agarose gel electrophoresis.

Determination of bcl-2, p 53 and bax by FACS. For the determination of the expression of bcl-2, p53 and bax proteins, splenic lymphocytes of mice (control or PA-treated) were permeabilized and fixed as described earlier. One part of the cells (1×10^6 cells) from each group was incubated with monoclonal anti-p53 antibody (1 μg/ml in PBS containing 1% BSA) for 1 h at room temperature, washed with three changes of PBS and then incubated with FITC-conjugated goat anti-mouse antibody for one more hour at room temperature. Other two parts of the cells (1×10^6 cells in each group) were incubated with either polyclonal anti-bcl-2 antibody (2 μg/ml in PBS containing 1% BSA) or polyclonal anti-bax antibody (1 μg/ml in PBS containing 1% BSA) for 1 h at room temperature, and then with FITC-conjugated goat anti-rabbit antibody. Cells were washed thoroughly and analyzed in a flow cytometer as described earlier. Histogram display of FL1-H (*x*-axis; FITC-fluorescence) versus counts (*y*-axis; number of cells) has been shown in logarithmic fluorescence intensity.

Cytokine assay. Cytokine enzyme-linked immunosorbent assay (ELISA) kits for IL-2, IFN-γ and TNF-α were used for this purpose. The assays were performed according to the manufacturer's protocol using serum samples collected at different time points and pooled together for each group. The cytokines were estimated thrice, each time by performing a separate set of experiments.

RESULTS

Binding of protein A to mice splenic B lymphocytes. As a prerequisite of studying the mechanism of PA action we first tested whether PA binds to splenic lymphocytes or not. Flow cytometric analysis using FITC-labeled PA showed that there is a dose dependent binding of PA to these cells as obtained by the gradual shifting of FITC-PA bound cells from the left to the right channels due to the increase in fluorescence intensity (Figs. 1A to 1F).

In vivo proliferation of splenic B cells. After obtaining the dose dependent PA binding to the splenic lymphocytes, we next examined the role of PA in splenic lymphocyte proliferation. Our results showed that at a dose of 1 μg PA/20 g mice (iv, twice a week for 2 weeks) splenic lymphocyte number was increased by ~5-fold over control but at 10 μg dose cell number was decreased in comparison to 1 μg dose (Fig. 1G).

Effect of protein A on cell-cycle phase distribution of nuclear DNA. The above results prompted us to find out why proliferation is inhibited at a higher dose. To get a clear picture of what is exactly happening at the DNA level, we studied the cell-cycle phase distribution of nuclear DNA. Flow cytometric data revealed that PA induced shifting of G0/G1 phases to S and G2/M phases of cell-cycle at a dose of 1 μg (Fig. 2A vs Fig. 2B). In this case, 37.8% cells shifted to S and G2/M phases from 18.1% cells in untreated cells indicating the mitogenic effect of PA at this dose. In contrast, at the dose of 10 μg, PA induced the formation of sub-G1-cell population as revealed by the increase in hypoploid ($<$2n) peak of cell-cycle DNA (from 0.5% in to 10.6% in Figs. 2A and 2C) indicating the breakdown of DNA at this dose of PA. These results suggested that PA exerts differential effects in immunocytes at different doses.

Protein A-induced oligonucleosomal fragmentation. To establish the reasons for DNA breakdown, we observed that unlike in control or at 1 μg PA dose (Fig. 3, lanes 1 and 2), 10 μg dose dramatically induces DNA

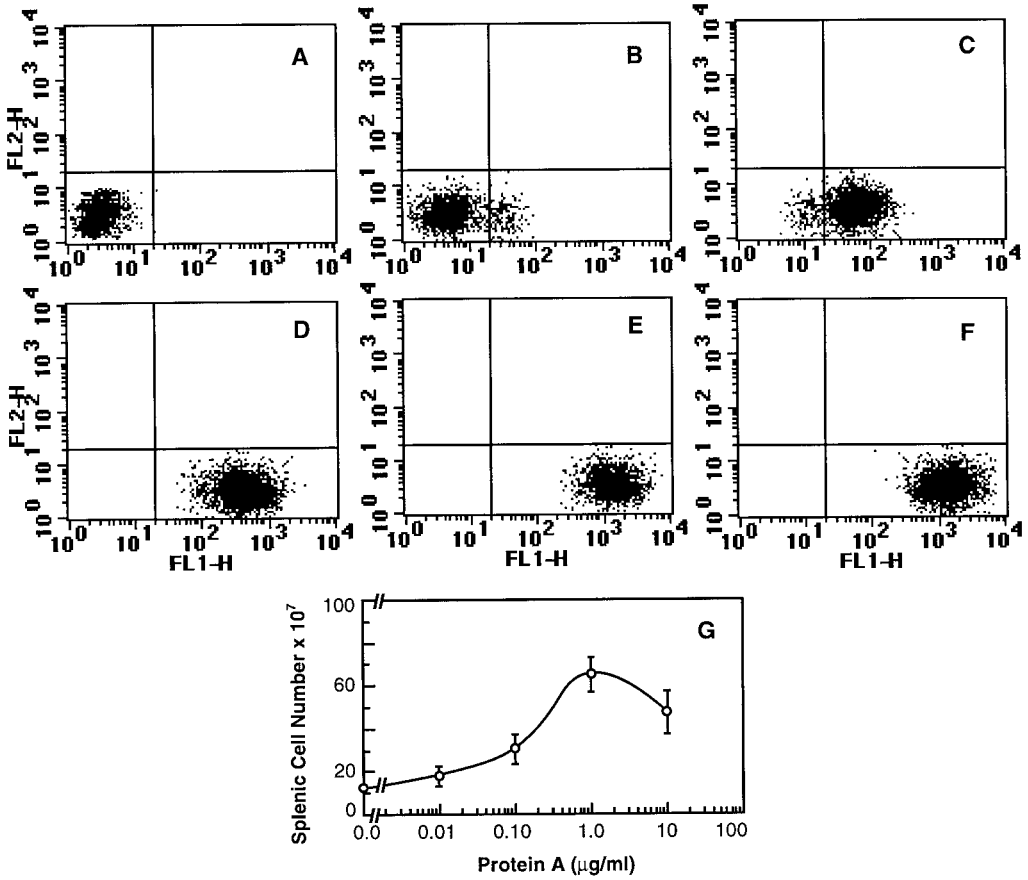

FIG. 1. Binding of protein A to mice splenic B lymphocytes. Splenic lymphocytes were incubated with (A) FITC alone or (B–F) different concentrations of FITC-conjugated protein A (0.01–100 μg/ml) for 30 min. Cells were fixed and washed followed by cytofluorometric analysis. Dot plot display of FL-1 H (*x*-axis; FITC-fluorescence) versus FL-2 H (*y*-axis) has been shown. The experiments were repeated thrice with similar results. (G) Effect of protein A on *in vivo* proliferation of splenic lymphocytes was determined as the number of viable cells (open circle). Data are expressed as the mean ± SEM of triplicate cultures.

fragmentation, as was evidenced by DNA ladder formation (Fig. 3, lane 3). These results supported the above observations and proved that PA induced proliferative effect at lower dose (1 μg) and apoptosis at a higher dose (10 μg).

Effect of protein A on cytokine production. The profiles of PA-stimulated cytokine productions were different at these two doses of PA. Results of Table 1 indicate that 1-μg dose of PA upregulates IFN-γ (5-fold) and TNF-α (12.5-fold) compared with untreated control. Concentration of IL-2 was very little at this dose. However, at 10 μg dose, increase was much more in cases of TNF-α (69-fold) and IL-2 (3-fold).

Expression of bcl-2, P53 and bax proteins as a consequence of protein A treatment. To find out the mechanism(s) of PA-induced apoptosis and immunopotentiation, we next examined whether different doses of PA have any differential effects on the expression of the pro-proliferative protein, bcl-2, and pro-apoptotic proteins, p53 and bax. Using flow cytometric technique, we observed that at 1 μg dose of PA, bcl-2 increased significantly in splenic cells (Fig. 4A vs Fig. 4B) but decreased at 10 μg dose (Fig. 4C). However, p53 level was low in the untreated host (Fig. 4D) and remained unaffected by 1-μg dose (Fig. 4E). Interestingly, the level of this pro-apoptotic protein, p53, increased significantly in these cells after the treatment with 10 μg PA (Fig. 4F). Similar effects were obtained on bax protein expression in these cells (Figs. 4G–4I).

DISCUSSION

Our observations described above show that PA at different doses induce different molecular mechanisms in the same cell type, ending with differential effects. The results show that although at lower dose PA stimulates cell proliferation, at the higher dose, formation of sub G1-cell population in the cell-cycle was induced. Moreover, DNA laddering was obtained as the markers of apoptosis. All these results led us to conclude that PA at lower dose stimulates cell proliferation and at higher dose results in apoptosis of the same cells.

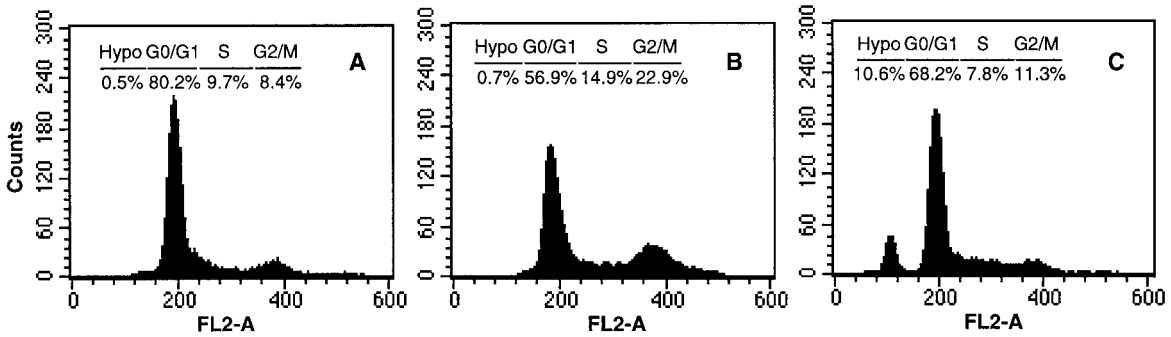

FIG. 2. Effect of protein A on cell-cycle phase distribution of nuclear DNA. Cell-cycle phase distribution of nuclear DNA of splenic lymphocytes of mice treated *in vivo* without (A) or with PA (1 or 10 μg/20 g mice; B or C) was determined flow cytometrically. Histogram display of FL2-A (*x*-axis, PI-fluorescence) versus counts (*y*-axis) has been displayed. The experiments were repeated more than three times with similar results.

It is acknowledged that for many but not all signals, the balance between the competing activities of the pro-proliferative/anti-apoptotic and pro-apoptotic proteins determines the fate of any cell. Among the genes linked to proliferation and apoptosis, bcl-2, bax, and p53 gene expressions assume much significance (21, 22). It was reported that induced overexpression of p53 may cause apoptotic death of cells (23). On the other hand, the survival function of bcl-2 is known to be opposed by its close relative bax (24). Interestingly, expression of bax is activated in some cell types by the p53 tumor suppressor, which can provoke apoptosis (25). It is thus envisaged that pro- and anti-apoptotic proteins titrate one another's function, suggesting that their relative concentration may act as a rheostat for the suicide program (24). Thus the opposing and interactive effects of bcl-2, bax and p53 as has been observed by various workers (26, 27) have relevance with our observations. In this case, PA at the dose of 1 μg/20 g mice induces the expression of the pro-proliferative protein bcl-2 and stimulates the proliferation of splenic lymphocytes as concluded from the results of Fig. 4. These results were further supported by the finding that bcl-2 gene is expressed in high level during normal lymphocyte proliferation (28, 29). The novelty, however, of the present observations lies in the differential effects of the same protein PA on splenic lymphocytes at different doses, i.e., an increase in bcl-2 gene expressions in these cells at 1 μg dose while increasing p53 and bax expressions in the same cell type at 10 μg dose. Thus, it indicated that the cross-talk between these pro- and anti-apoptotic proteins may contribute to maintain a balance between pro- and anti-apoptotic reactions.

It is interesting to note that even the endogenous immunomodulators, e.g., ILs, TNF-α, IFN-γ etc., beyond certain concentrations, induce apoptosis (30–32). PA has been found to upregulate the production of various cytokines. It is known that the relative concentration of IFN-γ and IL-2 is important factor in decid-

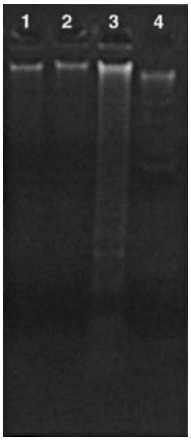

FIG. 3. Protein A-induced oligonucleosomal fragmentation. Splenic lymphocytes of mice treated with or without PA (1 or 10 μg/20 g body wt) were harvested and genomic DNA from equal numbers of cells (lane 1, PBS-treated; lane 2, 1 μg PA-treated; and lane 3, 10 μg PA-treated) were isolated and separated in 2% agarose by gel electrophoresis. Lane 4 is molecular weight marker. The experiments were performed three times with similar results.

TABLE 1

Effect of Different Doses of Protein A on Cytokine Production *in Vivo*

	Fold change over control	
Cytokine	1 μg protein A	10 μg protein A
IFN-γ	5	5
IL-2	[a]	3
TNF-α	12.5	69

Note. Blood was collected from mice treated with or without PA (1 or 10 μg/20 g body wt) and IFN-γ, IL-2, and TNF-α were estimated from the blood samples using ELISA kit. Data were represented as fold of cytokines increase over control.

[a] IL-2 level at this dose of protein A was so little that it was beyond the detection limit of the kit.

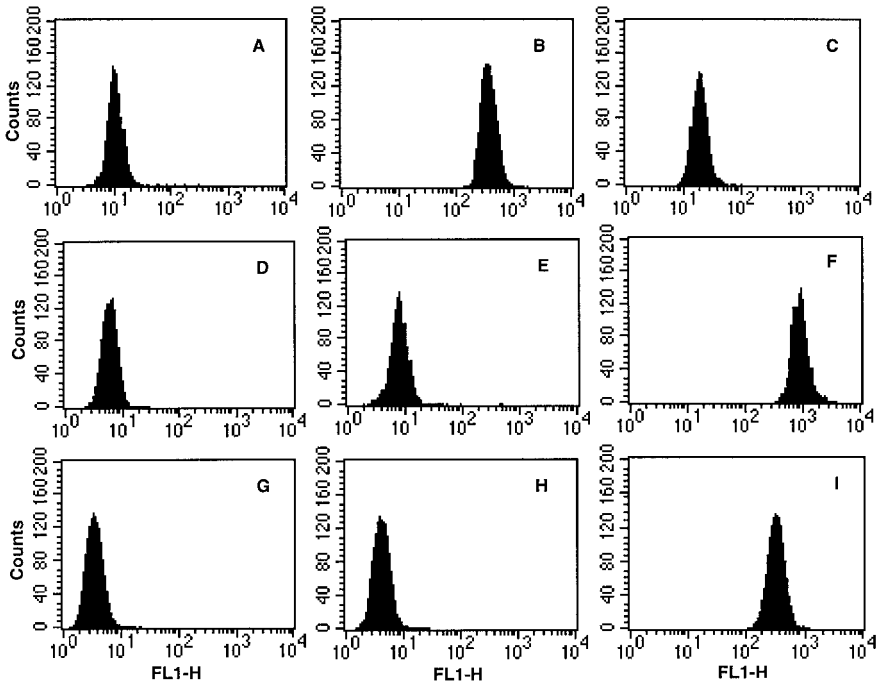

FIG. 4. Expression of bcl-2, p53 and bax proteins as a consequence of protein A treatment. Mice splenic lymphocytes were incubated with anti-bcl-2/anti-p53/anti-bax antibodies and then with FITC-conjugated second antibodies. The cells were analyzed on a flow cytometer. Histogram display of FL1-H (*x*-axis; FITC-fluorescence) versus counts (*y*-axis; number of cells) has been shown in logarithmic fluorescence intensity. Panels: (A) bcl-2 of control splenic lymphocytes (B) bcl-2 of 1 μg PA-treated splenic lymphocytes, (C) bcl-2 of 10 μg PA-treated splenic lymphocytes, (D) p53 of control splenic lymphocytes (E) p53 of 1 μg PA-treated splenic lymphocytes, (F) p53 of 10 μg PA-treated splenic lymphocytes, (G) bax of control splenic lymphocytes (H) bax of 1 μg PA-treated splenic lymphocytes, and (I) bax of 10 μg PA-treated splenic lymphocytes. The experiments were repeated thrice with similar results.

ing the promotion of lymphocyte proliferation or apoptosis (33). Since in our system, at lower dose only IFN-γ was increased keeping IL-2 stimulation nominal, the cells were directed toward proliferative pathway. However, at higher dose, an increase in IL-2 together with IFN-γ may have shifted the balance to the other side and supported apoptosis of these cells. This notion was further supported by the 69-fold increase in TNF-α at the higher dose of PA in comparison to 12.5-fold with 1 μg dose. Such a high level of this cytokine, which is a known apoptosis inducer, may be one of the important factors of normal cell apoptosis at higher dose of PA. All these findings suggest that the beneficial immunostimulatory dose of PA is 1 μg/20 g animal. PA has long been known as an immunopotentiator (6–16) and thus might be used as immunorestorer during immunodepressed condition as occur due to many diseases. However, the knowledge gathered from our study would help to select the proper dose of PA which otherwise may become detrimental for the immune system itself making the situation worse. On the other hand, it is recognized that during many diseases, e.g., autoimmune disease, desired apoptosis fails to take place (34). In those situation higher dose(s) of PA can be used as the part of therapy.

ACKNOWLEDGMENTS

Grateful acknowledgment is due to Professor G. C. Das of the University of Texas at Tyln, U.S.A., for the generous gift of p53 antibody. The authors thank Mr. U. Ghosh for technical assistance and Mrs. R. Das for secretarial assistance.

REFERENCES

1. Cohen, J. J. (1993) *Immunol. Today* **14,** 126–137.
2. Williams, G. T. (1991) *Cell* **65,** 1097–1098.
3. Boehm, U., Klamp,T., Groot, M., and Howard, J. C. (1997) *Annu. Rev. Immunol.* **15,** 449–495.
4. Mapara, M. Y., Bargou, R., Zugck, C., Dohner, H., Ustauglu, F., Jonker, R. R., Krammer, P. H., and Dorken, B. (1993) *Eur. J. Immunol.* **23,** 702–708.
5. Sveinbjornsson, B., Rushfeldt, C., Olsen, R., Smedsrod, B., and Seljelid, R. (1997) *Biochem. Biophys. Res. Commun.* **7,** 270–275.
6. Fiejo, G. C., Sabbaga, J., Carneiro, C. R., and Brigido, M. M. (1997) *Immunology* **91,** 479–485.
7. Tiedemann, R. E., and Fraser, J. D. (1996) *J. Immunol.* **157,** 3958–3966.
8. Langone, J. J., Dac, C., Bennet, D., and Terman, D. (1984) *J. Immunol.* **133,** 1057–1063.
9. Prasad, A. K., Singh, K. P., Saxena, A. K., Mathur, N., and Ray, P. K. (1987) *Immunopharmacol. Immunotoxicol.* **9,** 541–561.
10. Singh, K. P., Shau, M., Gupta, R. K., Kopald, K., and Ray, P. K. (1992) *Immunopharmacol. Immunotoxicol.* **14,** 79–103.

11. Dwivedi, P. D., Verma, A. S., and Ray, P. K. (1992) *Immunopharmacol. Immunotoxicol.* **14,** 105–128.
12. Ray, P. K., Dutta, P. K., Das, T., Srivastava, M., and Subbulakshmi, V. (1997) *in* Immunomodulation (Upadhyay, S. N., Ed.), pp. 101–110, Narosa, New Delhi, India.
13. Sinha, P., Ghosh, A. K., Das, T., Sa, G., and Ray, P. K. (1999) *Immunol. Lett.,* in press.
14. Goenka, S., Das, T., Sa, G., and Ray, P. K. (1998) *Biochem. Biophys. Res. Commun.* **250,** 425–429.
15. Ghosh, A. K., Sinha, P., Das, T., Sa, G., and Ray, P. K.(1999) *Biochem. Biophys. Res. Commun.,* in press.
16. Das, T., Sa, G., Subbulakshmi, V., Sen, P. C., Biswas, S., and Ray, P. K. (1999) *Immunopharmacol. Immunotoxicol.,* in press.
17. Sukla, Y., Verma, A. S., Mehrotras, N. K., and Ray, P. K. (1996) *Cancer Lett.* **103,** 41–47.
18. Ray, P. K., Raychaudhury, S., and Allen, P. (1982) *Cancer Res.* **42,** 4970–4970.
19. Subbulakshmi, V., Ghosh, A. K., Das, T., and Ray, P. K. (1998) *Biochem. Biophys. Res. Commun.* **250,** 15–21.
20. Ray, P. K., Dohadwala, M., Bandyopadhyay, S., Canchanapan, P., and McLaughlin, D. (1985) *Cancer Chemother. Pharmacol.* **4,** 59–62.
21. Pan, H., Yin, C., and Van-Dyke, T. (1997) *Cancer Surv.* **29,** 305–327.
22. Evan, G., and Littlewood, T. (1998) *Science* **281,** 1317–1322.
23. Pardhasaradhi, B. D., and Khar, A. (1996) *Indian J. Exp. Biol.* **34,** 945–948.
24. Oltvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1993) *Cell* **74,** 609–619.
25. Miyashita, T., and Reed, J. C. (1995) *Cell* **80,** 293–299.
26. Vollmer, R. K., Humphrey, P. A., Swanson, P. E., Wick, M. R., and Hudson, M. L. (1998) *Cancer* **82,** 715–723.
27. Hori, M., Nogami, T., Itabashi, M., Yoshimi, F., Ono, H., and Koizumi, S. (1997) *Pathol. Int.* **47,** 757–762.
28. Li, L., Krajewski, S., Reed, J. C., and Choi, Y. S. (1997) *Cell. Immunol.* **178,** 33–41.
29. Wilson, B. E., Mochon, E., and Boxer, L. M. (1996) *Mol. Cell. Biol.* **16,** 5546–5556.
30. Boehm, U., Klamp, T., Groot, M., and Howard, J. C., (1997) *Annu. Rev. Immunol.* **15,** 749–795.
31. Mapara, M. Y., Bargou, R., Zugck, C., Dohner, H., Ustauglu, F., Jonker, R. R., Krammer, P. H., and Dorken, B. (1993) *Eur. J. Immunol.* **23,** 702–708.
32. Sveinbjornsson, B., Rushfeldt, C., Olsen, R., Smedsrod, B., and Seljelid, R. (1997) *Biochem. Biophys. Res. Commun.* **7,** 270–275.
33. Leonardo, M. J. (1991) *Nature* **353,** 858–860.
34. Cory, S. (1995) *Annu. Rev. Immunol.* **13,** 513–543.