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S. aureus Superantigen Protein A Expands $CD4^+$/$CD8^+$/ $CD19^+$/$CD34^+$ Cells in Mice: A Potential Immunorestorer[1]

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Protein A (PA) of *Staphylococcus aureus* is known for its immunostimulatory, anti-cancer, and anti-toxic properties. The present study revealed that PA stimulates specific immunocytes to act as a potential immunorestorer. It has also been shown that the percentage of various cell types bearing different clusters of differentiation markers, e.g., $CD4^+$, $CD8^+$, $CD19^+$, increases considerably after inoculation with PA. It has also been observed that $CD34^+$ progenitor cells of bone marrow also increased significantly ($P < 0.05$) upon PA treatment. PA significantly elevated Th-1 cytokines, e.g., IFN-γ, TNF-α, and IL-1α. The increased percentages of $CD4^+$, $CD8^+$, $CD19^+$, $CD34^+$ cells and elevated cytokine levels in PA treated animals may contribute to the reported anti-tumor, anti-fungal, anti-parasitic, and anti-toxic properties of PA. Since in various diseased conditions and during toxic drug therapy lymphocytes bearing such differentiation markers get suppressed, this type of approach could help in immunorestoration of the host. These findings might help in designing therapeutic approaches toward various diseases which cause immunosuppression.

Key Words: **Protein A; cluster of differentiation; immunorestorer.**

Immunodepression is a major problem encountered during various therapy (1–3), which induces immunodeficiency syndromes (4), making the host susceptible to infection. Clinical management of such patients is difficult and poses life-threatening problems, including death (5–7). Apart from cancer patients (8), transplant recipients (9,10) patients suffering from in-born immunodeficiency disorders, and also during HIV-infection (11), the resulting immunodeficiency also compromises the successes of the treatment. A successful immunorestoration process, from its depressed status, would be enormously beneficial to immunocompromised patients. Therefore, search for an effective immune function restorer has been going on for many years. In fact it was evidenced that $CD34^+$ cells of bone marrow can reconstitute the hematopoiesis of primates, human, and mice undergoing autologus marrow infusion after myeloblastic therapy (12). It would be of much help if the progenitor $CD34^+$ cells could be expanded, especially in AIDS patients treated with anti-retroviral drug, zidovudine.

Protein A (PA), a 42 kDa protein, is present on the cell wall of *Staphylococcus aureus* Cowan-I. It is known to react with CH_2-CH_3 domains of the Fc structure of immunoglobulins and also bind immune complexes (13). PA has also been known as B-cell superantigen (14). It has been reported that PA can bind $VH3^+$ segment of Fab structure of immunoglobulins (15). Thus PA was suggested to stimulate B-cell receptor (BCR) and thus providing continuous stimulation for B-cell proliferation (16). PA has been reported to protect the host from toxic insults of many drugs and chemicals (17–21) and to regress tumors (22–26). Recently, PA has been shown to render protection against Candidiasis (27) and against *Leishmania donovani* infection (28). The structural uniqueness, physicochemical properties and immunological potential of PA have well been reviewed (28–29). PA is also known to stimulate CFU-E, BFU-E and CFU-GM in bone marrow (21). PA has been shown to be an effective inducer of IL1, IL2, IFN-γ, TNF-α and nitric oxide (NO) (31), which may have an autocrine function on the precursor cells of different lymphocyte lineages. Interestingly, in all studies with PA an overall proliferative response of immunocytes of spleen was noted. But no such report is available in the literature describing as to what type(s) of cells expand with specific differentiation markers, including the $CD34^+$ hematopoietic stem cells. Here we report that PA treatment expands the percentages of $CD4^+$, $CD8^+$, $CD19^+$ and $CD34^+$ cells and also elevates Th-1 cytokine levels simultaneously.

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MATERIALS AND METHODS

Chemicals. Protein-A was purchased from Pharmacia Fine Chemicals, Stockholm, Sweden. DNA cell-cycle kit was procured from Becton Dickinson, USA. Fluorescence conjugated anti-mouse monoclonal antibodies against $CD4^+$, $CD8^+$, $CD19^+$, and $CD34^+$ were purchased from Pharmingen. Cytokine ELISA kits for IL1α, TNFα, IFN-γ were procured from Genzyme, USA. Ficoll-hypaque was purchased from Sigma Chemicals, USA and RPMI-1640 medium was obtained from Gibco BRL. Phosphate Buffer Saline was obtained from Himedia, India.

Treatment of animals. Six to eight week old male Swiss albino mice, weighing 20-25 g were maintained on a commercial pellet diet. The animals were divided into two groups of five animals each and treated separately. i) Control (injected with sterile normal saline i.v.) and group. ii) PA group (injected with PA i.v., @ 1 μg/animal bi-weekly for 2 weeks. After 24 hours of last injection, the animals were sacrificed for the experiment.

Isolation of lymphocytes from spleen and thymus. Spleens from Swiss albino mice were removed aseptically and single cell suspensions were made in RPMI 1640 medium. Red blood cells were allowed to sediment by keeping the tubes containing cell suspension at 45 degree angle for 30 min at 4°C. The top supernatant containing leukocyte rich population was then allowed to adhere in petridishes at 37°C for 1 h. The non-adherent cell populations were collected and were subjected to Ficoll-hypaque density gradient separation. The buffy layer was collected, washed and used as the source of cells for further studies.

Preparation of PBMC from blood. Blood from both control and PA treated etherized Swiss albino mice were collected in heparinized tubes from retro-orbital plexus. The blood samples were subjected to Ficoll-hypaque density gradient centrifugation at 2000 rpm for 30 min. The buffy coat was collected as PBMC population.

Preparation of bone marrow cells. Femurs were asceptically removed from Swiss albino mice and bone marrow was flushed with a 26 gauge needle. A single cell suspension was made with repeated aspiration. The cells were resuspended in RPMI-1640 at a final concentration of 1×10^6 cells/ml.

Cell cycle analysis of lymphocytes by FACS Calibur. After harvesting lymphocytes from spleen, cell-cycle phase distribution of nuclear DNA were determined by FACS Calibur (Becton Dickinson), using a fluorescence (FL-2) detector equipped with a 585/42 band pass filter (linear scale Becton Dickinson) and using Cycle Test Plus DNA reagent kit (Becton Dickinson Immunocytometry System) cell-cycle phase distribution of nuclear DNA was determined using CellQuest Software and a total of 10,000 events were acquired for analysis (Simultest LeucoGATE was used to reduce debris, monocytes, granulocytes or other contamination, if any).

Flow cytometric study on $CD4^+/CD8^+$ specific cells of spleen, thymus, and PBMC. Lymphocytes from spleen and, thymus as well as PBMC of blood were taken separately in eppendorf tubes. Cells were washed properly with PBS to get rid of contamination. In each tube R-phycoerythrin (R-PE)-conjugated rat anti-mouse $CD4^+$ (L3T4) monoclonal antibody (1 μg/10^6 cells) and fluorescing isothiocyanate (FITC)-conjugated rat anti-mouse $CD8^+$ (Lyt-2) monoclonal antibody (1 μg/10^6 cells) were added. The tubes were then kept at 37°C in rocking condition for 1 hr in the dark. The cells were then washed with PBS and fixed with 1% *p*-formaldehyde for 1 h. The cells were then analyzed using FACS Calibur (Becton Dickinson, USA) equipped with 488 nm Argon laser light source and a 530 nm band pass filter for FITC fluorescence (FL-1H) and 575 nm band pass filter for PE fluorescence (FL-2H). Cells were gated properly and a total of 10,000 events were acquired and analyzed using CellQuest Software.

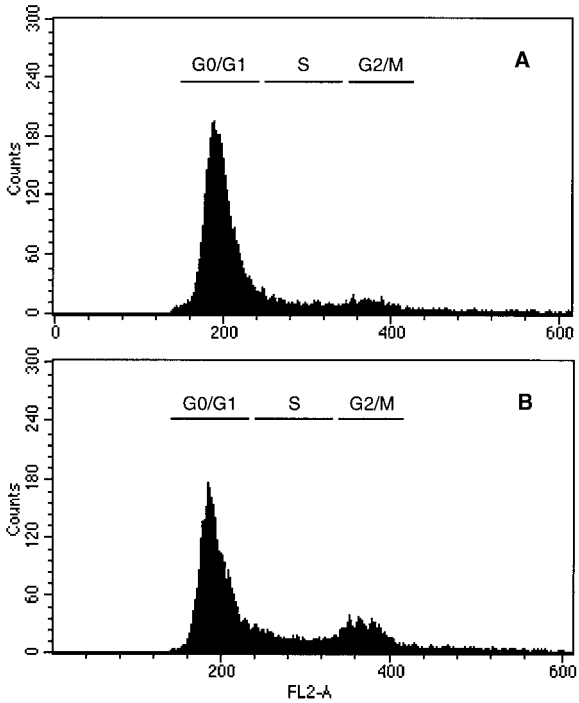

FIG. 1. Protein A-induced shift of cell-cycle phase distribution from G_0/G_1 to S and G_2M phase after *in vivo* PA treatment. The cells from untreated (A) and Protein A-treatment were permeabilized and nuclear DNA was stained with propidium iodide. Analysis of cell-cycle phase distribution of nuclear DNA was performed using FACS Calibur as described in Materials and Methods.

Flow cytometric study of $CD19^+$ cell of splenic lymphocytes. Splenic lymphocytes were taken in separate tubes and FITC-conjugated rat anti-mouse $CD19^+$ monoclonal antibody (1 μg/10^6 cells) was added. Control cells as well as PA treated cells were kept at 37°C in a rocker for 1 hour in dark. The cells were then washed with PBS and fixed with 1% paraformaldehyde for 1 h. The cells were again washed, and analyzed in FACS Calibur using 530 nm band pass filter for FITC fluorescence (FL-1H) as mentioned above. Cells were gated properly total of 10,000 events were acquired and analyzed using CellQuest Software.

FACS analysis of $CD34^+$ cells of bone marrow. Bone marrow cells were incubated with FITC-conjugated rat anti-mouse $CD34^+$ monoclonal antibody (1 μg/10^6 cells) for 1 hr in dark. Cells were washed with RPMI-1640 medium and fixed with 1% paraformaldehyde 1 h. The cells were then washed with PBS and analyzed in FACS Calibur as mentioned above.

Cytokine assay. The serum samples were collected at different time points and pooled together for each group. $IL_1\alpha$, TNF-α and IFN-γ (The Th-1 cytokines) contents in serum were assayed using ELISA kits according to the manufacturer's protocol (Genzyme, USA). The assays were done thrice each time performing separate set of experiments.

RESULTS

Cell-cycle analysis of splenic lymphocytes from mice treated with PA. Flowcytometric data (Fig. 1) revealed that PA induced shifting of cells from G_0/G_1 phases to S and G_2/M phases of the cell-cycle. In comparison to the untreated cells *in vivo* protein-A treat-

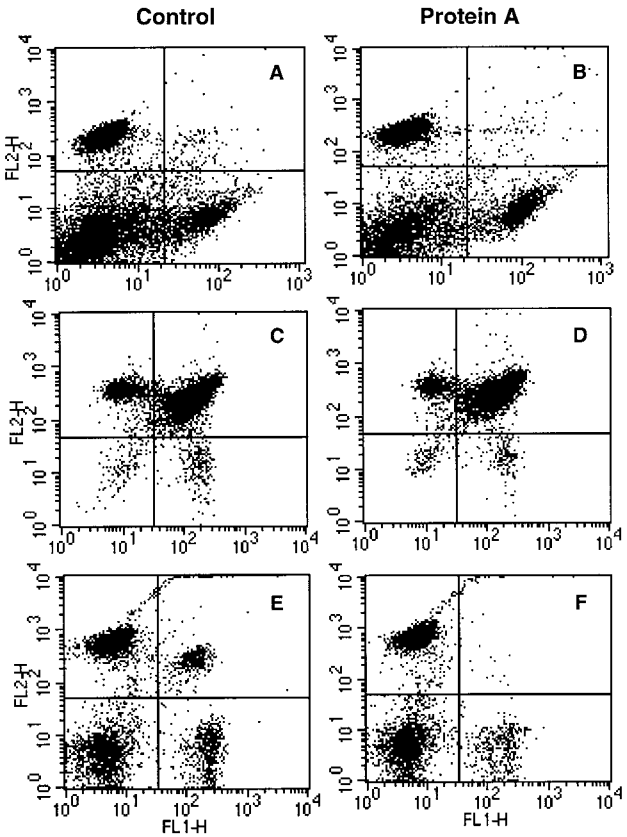

FIG. 2. Effect of Protein A on $CD4^+$ and $CD8^+$ cell expression in mice. Panels A and B show the percent distribution of $CD4^+$ (FL2-H) cells in control (A) and PA treated (B) mice splenic lymphocytes. FL1-H represents the percent of $CD8^+$ cells. Panels C and D show the $CD4^+/CD8^+$ cell distribution in thymus where the $CD4^+$ $CD8^+$ double positive cells are shown in the upper right (UR) quadrant. Panels E and F show the $CD4^+$ and $CD8^+$ PBMC distribution in control (E) as well as PA (F) treated mice. FL1-H indicates fluorescence (in logarithmic scale) due to FITC-conjugated $CD8^+$ cells, and FL2-H indicates fluorescence (in logarithmic scale) due to R-Phycoerythrine-conjugated $CD4^+$ cells.

ment, causes shifting of phase distribution of cell-cycle DNA from 19.7% S & G_2M phases to 45.3%.

Effect of PA on $CD4^+/CD8^+$ cells. Flowcytometric analysis of splenic lymphocytes showed that the percentage of $CD4^+$ was increased from 27.34% (control group, Fig. 2A) to 48.49% (PA-treated group, Fig. 2B) as a result of PA treatment. The number of $CD8^+$ cells was 17.5% (in control group, Fig. 2A) and 21.6% (PA-treated group, Fig. 2B). So the percentage of both $CD4^+$ and $CD8^+$ cells in splenic lymphocyte increased significantly as a result of PA treatment.

Cytofluorometric data also showed that most of the lymphocytes in the thymus were $CD4^+$ and $CD8^+$ (double positive). This double positive cell percentage was increased considerably in case of PA treated group (control 80.78%, Fig. 2B. PA treated group 84.70%. Fig. 2C). In the case of $CD4^+$ cells the percentage of cells also showed an increase from 88.44% (control group) to 93.19% (PA treated group).

As depicted in Figs. 2E and 2F the total percentage of $CD4^+$ cells in control animals was 51.89% and the percentage of this cell type in PA treated animals increased to 59.35% in PBMC. On the contrary the percentage of $CD8^+$ cells has decreased from 12.05% to 6.95% in case of PA treatment indicating reduction of $CD8^+$ cells in the circulation.

Role of PA on $CD19^+$ splenic B-lymphocytes. Our flowcytometric data showed that in PA-treated mice the percentage of $CD19^+$ cells in the spleen was much higher than that of the untreated mice. The $CD19^+$ cells increased to 40.73% in PA-treated group from 33.67% in untreated group (Table 1).

Effect of PA on bone marrow $CD34^+$ cells. Data of Table-1 showed that the percentage of *$CD34^+$* cells in the control group was 9.56 and in PA treated group 12.89%. Thus PA stimulates the *$CD34^+$* cells in the bone marrow.

Effect of PA on Th1 cytokine release. Data in Table 2 depicted that IFN-γ secretion started increasing after 2h of PA treatment and reached to its peak at 4h (4 folds). Thereafter, it declined and reached to the background level by 24h. TNF-α started increasing within 2h of PA treatment and reached to its peak within 4h. TNF-α increased by 12 folds in comparison to the control group. $IL_1\alpha$ also started increasing within 2h and reached to its peak in 4h. Here we also observed that $IL_1\alpha$ increased by 3 fold in PA treated mice.

DISCUSSION

On the basis of earlier studies various factors seem to contribute to the anti-tumorigenic potential of Protein A. Apart from its anti-tumor activity, PA is known

TABLE 1

Effect of Protein A on the Expression of $CD19^+$ and $CD34^+$ Cell Types

		Percentage cell populations bearing surface marker	
Cells	Cell type	Control group	PA-treated group
Spleen	$CD19^+$	33.67	40.67
Bone marrow	$CD34^+$	9.56	12.89

Note. Splenic lymphocytes and bone marrow cells from untreated or Protein A-treated Swiss albino mice were isolated. Cells were labeled with FITC conjugated anti-$CD19^+$ and FITC conjugated anti-$CD34^+$ monoclonal antibodies separately. Cells were fixed and used for flow cytometric analysis as described in Materials and Methods. A total of 10,000 events were acquired and data were analysed using CellQuest software.

TABLE 2

Effect of Protein A on the Th-1 Cytokine Release

Cytokines	Fold increase over control	
	Control	Protein A (1 μg/mouse)
IFN-γ	1	4
$IL_1\alpha$	1	3
TNF-α	1	12

Note. Swiss albino mice were treated with 1 μg of Protein A biweekly for 2 weeks. After the last injection serum samples (from control as well as PA-treated group) were collected at different time points. IFN-γ, IL-1α, TNF-α contents in serum were assayed using ELISA kits as described in Materials and Methods. Fold increase of cytokines release over untreated control has been shown.

to be an immunostimulant (32–33). PA also ameliorates the toxicity of various xenobiotics viz. cyclophosphamide, carbon tetrachloride, benzene, azathymidine (17–21), bacterial endotoxins (34) etc. Besides, in the recent past we have reported that PA has antifungal (27), and antiparasitic properties (28). Various laboratories showed that PA can function as both T and B cell mitogen and as a B cell superantigen (14).

It is possible that at least some of the observed properties of PA mentioned above must have been mediated by its effects on the proliferation and differentiation of various immunocytes. In the present study we have demonstrated that there is a G0/G1 to G_2M phase shifting of the splenic lymphocytes, which corroborates with the previous reports regarding the mitogenic potential of PA.

This report describes, for the first time, the effect of PA on the expression and expansion of various lymphocytes, having specific differentiation markers. The $CD4^+$ and $CD8^+$ cells are increased significantly ($P < 0.05$) in spleen, and also considerably in the thymus and PBMC. An increase in the percentage of $CD4^+$/$CD8^+$ cells in the thymus indicated T cell mitogenic potential of PA. So the protection against various pathogens, fungal infection (27), tumors (22–26) and even restoration of depleted immunocytes due to various toxic chemicals (17–21) may be related to this ability of PA. An increase in the Th-1 cytokine levels also corroborates with much higher percentages of $CD4^+$, $CD8^+$ $CD19^+$ $CD34^+$ cells of the immune system. Studies on $CD19^+$ cells indicated an appreciable proliferative response of B-lymphocytes. Larger the number of antibody producing B-lymphocytes, the more effective will be the antibody production.

One of the major drawbacks in the field of chemotherapy and radiation therapy is that they are mainly myelosuppressive (1–3). The drugs used selectively knockout the progenitor cells ($CD34^+$) of bone marrow. Here we found that the percentage of $CD34^+$ cells can be increased considerably by PA. During AIDS, the disease itself as well as the drug AZT. (3′-azido-3′-deoxythymidine) cause severe myelosuppressions (11) PA treatment in combination with AZT may be of immense help in ameliorating the toxicity as well as might increase the number of both $CD4^+$ and $CD34^+$ cells which are destroyed by HIV itself, and during AZT treatment. Extensive studies are going on in our laboratory with AZT and PA treatment of mice and our data corroborates such a hypothesis (Ghosh et al., 35).

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