Biochemical and Biophysical Research Communications **258,** 141–147 (1999)
Article ID bbrc.1999.0363, available online at http://www.idealibrary.com on IDEAL®

A Minimized Fc Binding Peptide from Protein A Induces Immunocyte Proliferation and Evokes Th1-Type Response in Mice[1]

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Received January 28, 1999

It is now well established that PA is a potent biological response modifier, showing simultaneously antitumor, antitoxic, anticarcinogenic, antifungal, antiparasitic and immunomodulatory properties. Since PA is a foreign protein, it is quite logical to assume that it may be cleaved into smaller peptide fragments *in vivo* which may be responsible for biological activities of whole PA molecule. The present study was undertaken to dissect out the structural entities of PA responsible for its biological properties. Protein A (PA) of *Staphylococcus aureus* has a unique property of binding with immunoglobulins. On the basis of molecular modeling and energy minimization studies a 20-mer tryptic fragment (theoretical) was predicted to retain IgG binding capacity which has been verified by immunoblot. This peptide sequence was selected to carry out experimental studies to show its functional mimicry of PA. We observed in the sera of 20-mer peptide treated mice that the concentrations of IFNγ, TNFα and IL1α increase to a peak level by 4 h; on the other hand, there was a decrease in IL4, IL6 and IL10 concentrations at the same time (4 h). The ratio of IFNγ to IL4 showed Th1 type of response with the peptide as well as with that of PA. The nitric oxide concentration in sera also increases and the peak increase was in 6 h with both the peptide and PA. Cell cycle analysis using FACS shows that 20 μg dose of peptide was non-toxic to thymocytes and spleenocytes; on the other hand, it was immunoproliferative, shifting the thymocytes and spleenocytes from G0/G1 to S phase of the cell cycle. Further studies are in progress to evaluate other biological properties of the peptide, to evaluate if this peptide could be used as a substitute of PA to mimic at least some of its biological activities. © 1999 Academic Press

Key Words: **protein A; Fc binding; cytokines; Th1/Th2 pathway; immunomodulation.**

Protein A (PA) was discovered from the cell wall structure of *Staphylococcus aureus*, and utilized for a long time in the laboratory for isolation and purification of antibody molecules, specially IgG because of its Fc binding property (1). This unique Fc binding property of PA was also utilized to remove the "blocking factors" from human cancer patients (2). Along with IgG binding affinity, diverse array of biological functions of PA were also demonstrated, such as antitumor (3, 4), antitoxic (5), anticarcinogenic (6), immunomodulatory (7) antifungal (8) and antiparasitic effects (9). Protein A has been demonstrated to act as B and T cell mitogen (10, 11). It also induces production of different cytokines (7), which are well known biological modulators regulating cellular growth and differentiation (12) on one hand, and apoptosis (13) on the other. Previous observations from our laboratory (5, 6) have demonstrated the antitoxic and anticancer properties of PA. Moreover, PA has been demonstrated to activate phase I and phase II biotransformation and detoxification enzymes, thus abrogating the toxicity of different toxic and carcinogenic chemicals (14, 15). During these studies (16) we have consistently observed that PA treatment leads to an increase in both peripheral blood and spleenic lymphocyte population. PA induced activation of cell cycle shifting *vis a vis* proliferation in non-Hodgkin's lymphoma has also been reported (17).

The B-domain of protein-A, a 56 residue domain, binds the Fc portion of IgGs with dissociation constant (Kd) of about 10-50 nM (1). X-ray (18) and NMR (19) studies showed that binding contacts are presented from helix 1 (Lys7-His18) and helix 2 (Glu25-Asp36) of the B-domain (PA).

It has been observed in recent years that in order to effect a biological function, large molecular structure of macromolecules may not be required always. The biological function may be mediated through a portion of the whole molecule, normally called the active site or by a proteolytically degraded small peptide molecule. A particular type of contour, a globular structure, helical

[1] This work was supported by DBT, Government of India, by awarding a postdoctoral fellowship to Dr. Pratima Sinha.
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0006-291X/99 $30.00

forms, alpha or beta structure, etc., still retained by a small molecule may be enough for mediating different types of biological functions.

Our hypothesis is that PA, being a foreign protein, may be subjected to proteolytic cleavage *in vivo*. It is thus possible that such degraded products might be functionally operative *in vivo*. The smaller functional versions would become synthetically accessible. Therefore theoretical tryptic fragments of all five IgG binding domains of PA were compared for their IgG binding capacity using computer mediated molecular modeling technique. We have chosen one of these tryptic fragments, a 20-mer peptide, where tbe best interaction was obtained with Fc. Comparison was made with the interaction taking place for B domain-Fc complex where crystal structure is available. For experimental studies IgG binding was verified by Immunoblot experiment. To find out whether this fragment can induce some of the immunomodulatory properties as are induced by PA molecule, the present study was undertaken. A substitute peptide retaining some specific properties of PA molecule would be very useful for its practical use.

MATERIALS AND METHODS

Reagents. Freeze dried protein A (5 mg/vial) was procured from Pharmacia fine Chemicals, Sweden. Human IgG and peroxide conjugated antihuman rabbit IgG were procured from Sigma Chemicals, USA. 20 aminoacid long synthetic peptide was synthesized from Genmed Synthesis Inc, USA. The purity of the peptide was checked by HPLC. All the other chemicals used in the study were of analytical grade purity. Cytokine ELISA kits were procured from Genzyme, USA. Cycle TEST PLUS DNA reagent kit was procured from Becton Dickinson Immunocytometry System, USA.

Molecular modeling and energy minimization. "Peptide map" program available in Wisconsin Sequence analysis package (GCG ver 8) was used to generate the peptide fragment of PA (five IgG binding domain) (20). The computer graphics analysis was performed by displaying the domain B-Fc structure using the software INSIGHT II (Biosym USA) and the 1fc2 coordinates (18) in the PDB Brookhaven database. To evaluate the interaction energies of many orientations of peptides relative to IgG (Fc region), while searching for the orientation that results in the lowest interaction energy, Docking type calculation were used (21). This includes single point interaction energy calculation varying the mutual orientation of Fc and the peptide fragments. The orientation of peptide and IgG (Fc) complex, which gave a low initial interaction energy, was subjected for energy minimization using DISCOVER (Biosym technologies, Inc.). Typically 100 steps of steepest descent followed by 2000 steps of conjugate gradient minimization were performed, after which the incremental decrease in energy was always <0.01 kj/mol and the rms derivative 0.01 kj/mol/A. Nonbounding interaction energy, in terms of Van der Waal interaction and electrostatic interaction was evaluated using CVFF (22) force field. The structure of peptide-Fc complexes were compared with the native B domain-Fc complex (crystal structure).

Activation of nitrocellulose and dot immunoblotting of peptides. Nitrocellulose paper was activated by the procedure described by Lauritzen *et al* (23) and the activated nitrocellulose paper was labelled as Nit-CHO. The 20-mer peptide was diluted in PBS to give concentration of 5.0, 10.0 and 20 μg/2 μl and dotted on Nit-CHO; parallely 1 μg/2 μl PA and 1 μg/2 μl BSA was also spotted on Nit-CHO as positive and negative controls respectively. The Nit-CHO membrane was then heated at 45°C for 15 min for dot immunobinding of peptides (23). The membrane was then processed for immunoblotting as per standard procedure (23).

Animals. Random bred, Swiss albino mice (male, 15-20 g body weight) were obtained and kept in group of 10 animals per polypropylene cage, under controlled temperature (20-25°C) and humidity (65-75%) with 12/12 h light/dark period. Animals were fed a synthetic pellet diet (Lipton India Ltd.) containing all essential nutrients freshly obtained and stored under standard conditions and water was given *ad libitum*.

Synthetic peptide or Protein A treatment. Synthetic peptide or Protein A was administered intraperitoneally in 100 μl saline at a dose of 20 μg/animal or 1 μg/animal respectively, twice weekly for two weeks. The dose of peptide was selected from a dose response study where 20 μg peptide showed IgG binding. Since 1 μg/animal dose of PA provides maximum immunoprotection (7) and abrogation of chemical toxicity (24), this standard dose was used. The control animals received only saline in the same schedule. After the last injection blood was collected at different time intervals (2 h, 4 h, 6 h, 24 h and 48 h). 5-7 mice were bleed for each time point, serum was separated and then polled for each time point separately for cytokine and NO assay. We also collected thymus, spleen of the same mice 24 h after last dose of PA to understand the cell cycle pattern.

Cytokine assay. Cytokine enzyme-linked immunosorbent assay (ELISA) kits for IFNγ, IL4, IL6, IL10, IL1α and TNFα were purchased from Genzyme, USA and the assays were done according to the manufacturer's protocol using the serum samples collected at different time point, after the last dose of peptide or PA. The cytokine was estimated thrice, each time by performing separate set of experiments and representative of the results are shown. We also collected thymus and spleen of the same mice 24 h after last dose of PA or peptide to check any toxic effect of the same.

Nitric oxide colorimetric assay. Nitrogen monoxide (NO) was determined in serum samples collected at different time interval via nitrate on microtiter plates using Boehringer Mannheim kit. Briefly nitrate present in the samples is reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of the enzyme nitrate reductase. The nitrite formed reacts with sulfanilamide and N-(1-napthyl)-ethyl-enediamine dihydrochloride to give a red-violet diazo dye. The diazo dye is measured on the basis of its absorbance in the visible range at 550 nm.

Cell cycle analysis by FACS calibur. Thymocytes and spleenocytes were collected from the same mice 24 h after the last dose of peptide or PA treatment as stated above. All the cells were processed separately using Cycle TEST PLUS DNA reagent kit (Becton Dickinson Co.) for cell cycle analysis according to manufacturers guideline. Cells were trypsinized, membranes were disrupted and the nuclear DNA was labeled with the flurochrome propidium iodide (PI; 150 mg/ml). Cell cycle phase distribution of nuclear DNA of untreated as well as peptide and PA treated cells were analyzed flowcytometrically in FACS Calibur, Fluoresence (FL-2) detector equipped with a 582/42 ban pass filter was used (linear scale, Becton Dickinson Co., San Jose, CA). Total 20,000 events were acquired and analysis was performed using Cell Quest Software (Becton Dickinson Co.).

RESULTS

Molecular modeling. Theoretical prediction on tryptic digestion gives three peptide fragments (20 aa, 14 aa and 8 aa) from B domain, among which 20 aa peptide showed higher interaction than that of other fragments. 20 aa peptide could be formed from other do-

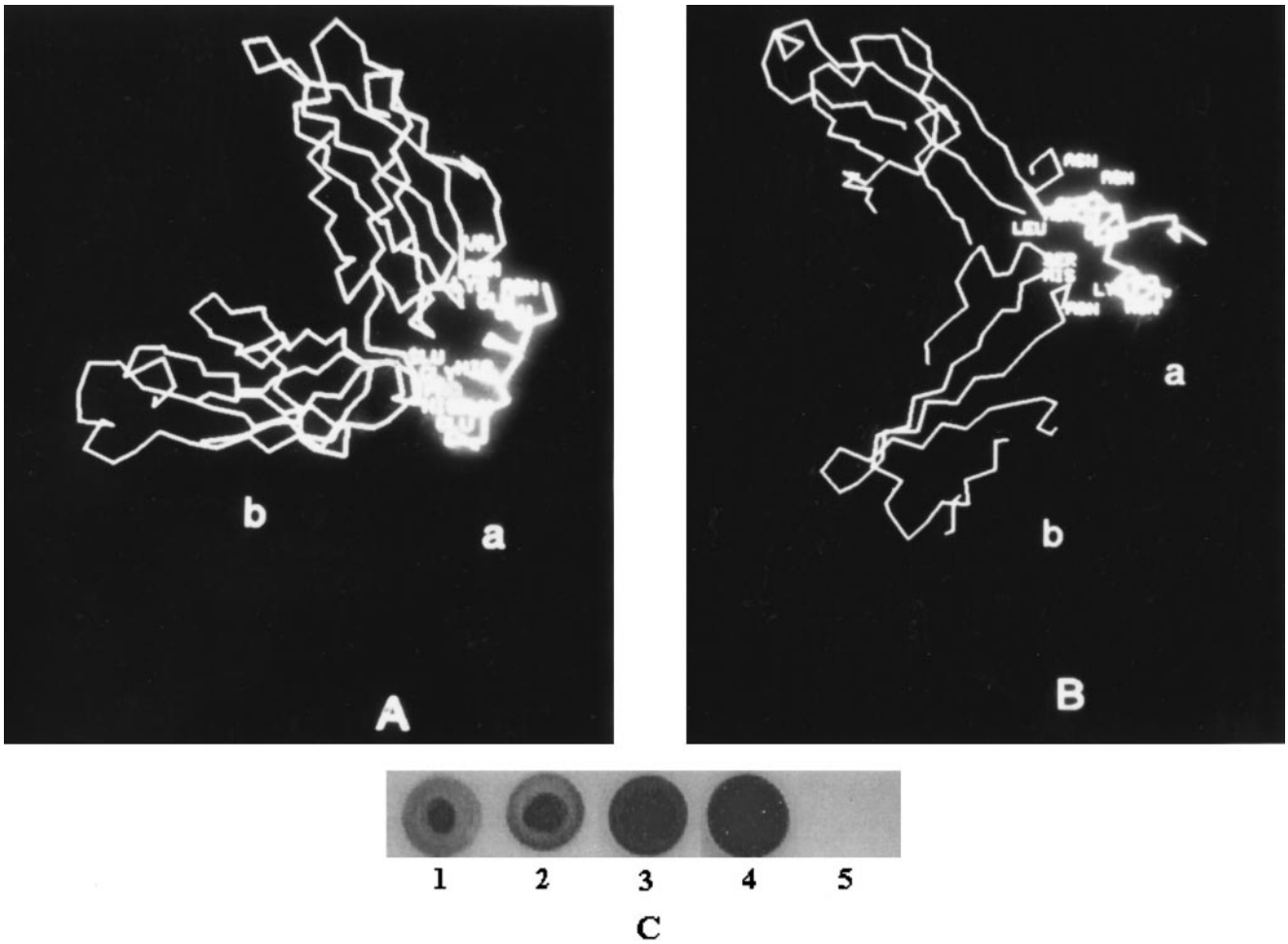

FIG. 1. Stereo view of Cα atom backbones of minimized energy conformation of IgG (Fc) (b) complexed with the following: (A) The tryptic fragment, 20-mer peptide (a) (B) B-domain of PA (a). Residues involved in hydrogen bond formation are marked. (C) Immunoblot of 20-mer peptide and PA, (1-3) 20-mer peptide at the dose of 5 μg, 10 μg and 20 μg respectively, (4) 1 μg PA as positive control (5) 1 μg BSA as negative control.

mains (D,A,C) too, except E: EQQNAFYEILHLPNLNEEQR (B-domain); EQQNAFYEILNMPNLNEEQR (A-domain) 2 aa difference; DQQSAFYEILNMPNLNEAQR (D-domain) 5 aa difference; EQQNAFYEILHLPNLTEEQR (C-domain) 1 aa difference. The comparison was based on B-domain fragment and an aminoacid was underlined when found different.

Among these 20-mer peptides from different IgG binding domain, B-domain fragment showed highest interaction with Fc of IgG from Van der Waal and electrostatic interaction energy point of view (Van der Waal interaction: −83.1 kcal/mol; Electrostatic interaction: −36.3 kcal/mol). A comparison was made with the interaction energies between the B domain and Fc (Van der Waal interaction: −131.7 kcal/mol; Electrostatic interaction: −39.9 kcal/mol) where available crystal structure was wild type conformation which was further minimized.

Figures 1 A & B shows stereo view of Cα tracing of IgG bound B domain (PA) and IgG bound 20-mer peptide (sequence used for experimental purpose). Aminoacids involved in hydrogen bond formation are displayed.

Immunoblot. The 20-mer peptide generated a strong brown color in the immunoblot experiment with IgG. Protein A (positive control) also showed obvious positive reaction. In case of BSA (negative control) no color was seen (Fig. 1C). This data clearly indicates that the 20-mer peptide can bind IgG molecules in a dose dependent manner, and the 20 μg dose gave the best color reaction (intensity), so this dose was chosen for other biological experiments.

Cytokines. We observed in the sera of mice that IFNγ, TNFα and IL1α starts increasing in 2 h and reaches to peak in 4 h, then it starts declining and reaches to basal level in 24 h. The fold increase in peak phase (4 h) of IFNγ, TNFα and IL1α was 4, 6 and 2 folds respectively with 20-mer peptide, whereas the increase was 5, 12 and 3 folds respectively with PA as

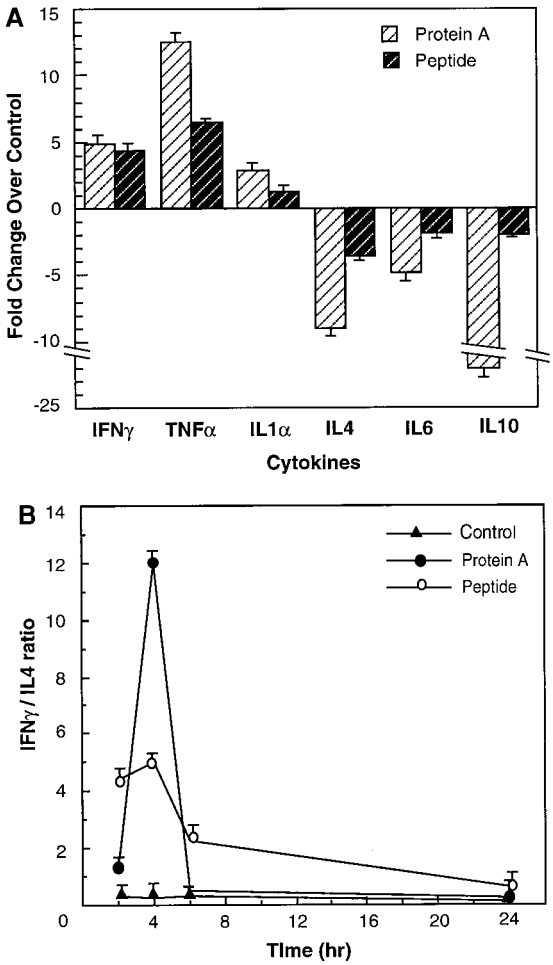

FIG. 2. Effect of 1 μg Protein A/animal or 20 μg Peptide/animal and saline control, twice weekly for two weeks, to swiss albino mice. Blood was collected at different time interval after last dose. Cytokine (IFNγ, TNFα, IL1α, IL4, IL6 and IL10) secretion profile was determined at different interval by ELISA. (A) Fold increase of cytokines over control at peak time period (4 h) (B) Time kinetics of IFNγ to IL4 ratio. Data shown here are are mean ± SEM of three sets of experiments.

compared to the back ground controls. In case of IL4, IL6 and IL10 we found an opposite trend, the cytokines starts declining in 2 h and the peak decline was in 4 h then reaches back to basal level in 24 h. Here the 20-mer peptide induced decline in IL4, IL6 and IL10 was 4, 2 and 2 folds respectively, whereas the decline was 9, 5 and 22 folds respectively with PA as compared to the background controls (Fig. 2A).

Peptide treatment to mice results in high levels of IFNγ as compared to IL4 resulting in IFNγ to IL4 ratio (110 pg/ml to 25 pg/ml) that was disproportionate to such ratios detected in serum of control mice (22 pg/ml to 50 pg/ml) during peak period of peptide stimulation (4 h). Thus IFNγ to IL4 ratio increases from 0.4 to 4.0 in peptide treated mice in peak phase (4 h), whereas the ratio was 12 (115 pg/ml to 10 pg/ml) in PA treated mice (Fig. 2B).

NO. It was observed that 20-mer peptide induced NO production *in vivo*, which was detectable 2 h after peptide treatment and reached to peak at 6 h, with a value 1.5 fold (62 μM) of the basal level (41 μM). Thereafter the NO in the serum plunged back to its control level (41 μM) 24 h after peptide treatment. The similar trend was observed in PA treated group where there was 2 fold increase in 6 h (Fig. 3).

Cell cycle. Our flowcytometric data showed that using both peptide and PA, the cell cycle pattern of thymocytes (Fig. 4 A,B,C) and spleenocytes (Fig. 4 D,E,F) shifted from G0/G1 to S phase indicating an increase in proliferative response of these cells. This shift in thymocytes were much higher in case of peptide (24%) as compared to that of PA (5%). There were negligible population of cells on the left side of G0/G1 indicating no apoptosis with peptide and PA to thymocytes (Fig. 4 A,B,C) as well as spleenocytes (Fig. 4 D,E,F). Therefore both peptide and PA caused proliferative response in both the cell types without causing any harm to these cells.

DISCUSSION

In the present study, we identified a 20-mer peptide fraction derived from theoretical tryptic digestion of PA, reflecting the sequence of the four Fc binding regions of IgG (D, A, B, C) except the E regions of PA, among the five homologous IgG binding domains of Protein A (20). We have observed that this 20-mer peptide fragment of B-domain has IgG binding property (25). We have now experimentally substantiated

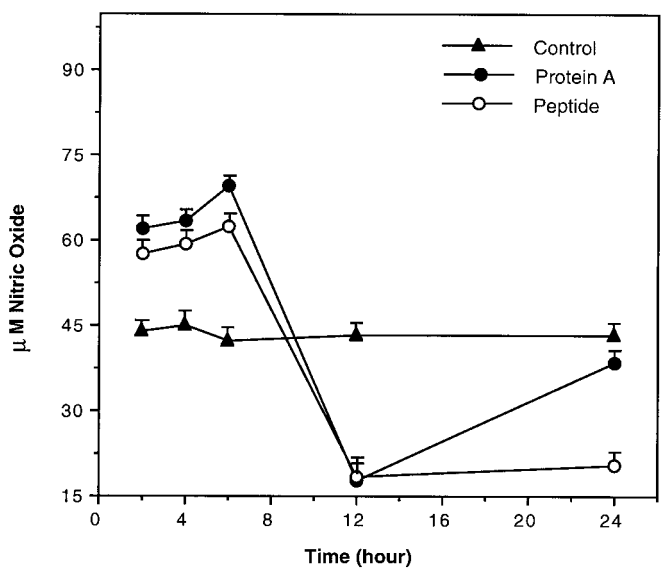

FIG. 3. Protein A or 20-mer peptide induced NO production *in vivo.* Protein A or peptide treatment schedule was same as described in Fig. 2. Time kinetics of nitric oxide level in serum is shown. Data shown here are mean ± SEM of three sets of experiments.

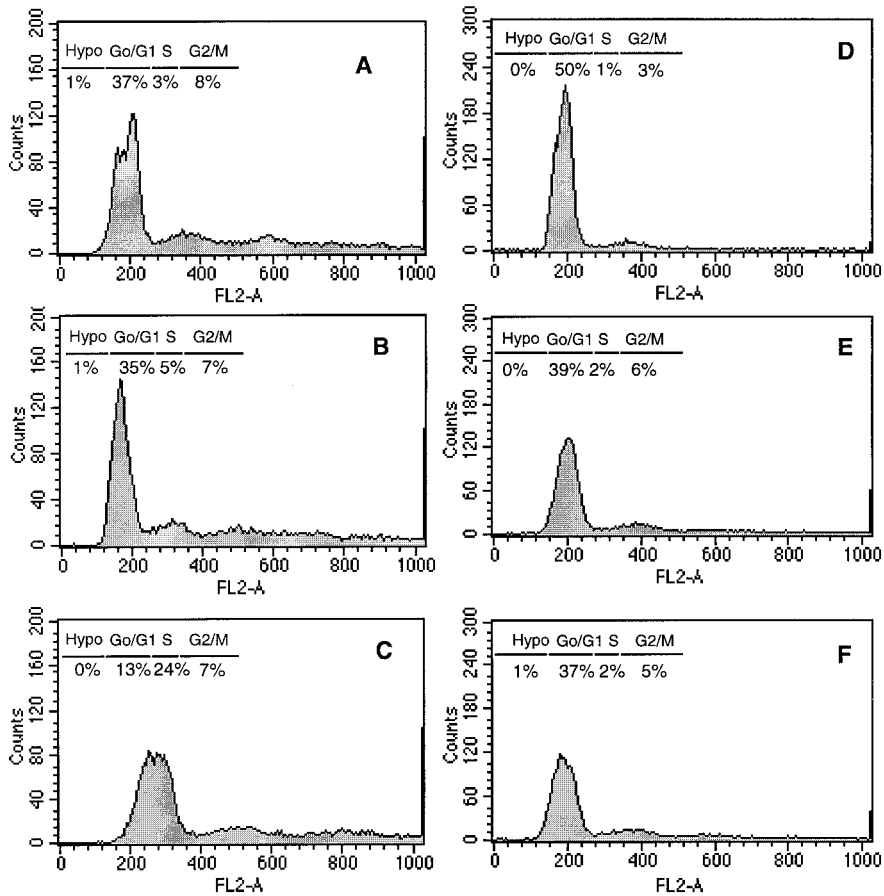

FIG. 4. Cell cycle phase distribution of thymocytes (A,B,C) and spleenocytes (D,E,F) 24 h after last dose of saline (A,D), 1 μg PA/animal (B,E) or 20 μg Peptide/animal (C,F). Protein A or peptide treatment schedule was same as described in Fig. 2. Thymus and Spleen were collected 24 h after last dose. Cells were permealized, nuclear DNA were stained with propidium iodide and analysed for cell cycle phase distribution by flow cytometry. Lymphocyte enriched regions were gated and the data were analysed with the use of Cell Quest Software.

such IgG binding capacity of this 20-mer peptide by immunoblot experiment, and simultaneously described that the peptide upregulates the production of IFNγ, TNFα and IL1α, whereas IL4, IL6 and IL10 productions are downregulated. The ratio of IFNγ to IL4 indicates clearly that the peptide evokes Th1 response as that of Protein A (7). The peptide also increases the level of nitric oxide in the serum as that of PA (26). It has also been observed that this dose of the peptide (20 μg/animal) was not toxic to the immunocytes but was immunoproliferative.

It was established for a long time that one of the binding site of PA is located in the Fc region of immunoglobulins (1). The crystal structure of the B domain complex with the Fc region is also available (18). Although PA has been recently declared as B-cell superantigen for its Fab binding property (27), the crystal structure of Fab is not yet available. In the present study, we did the theoretical modeling studies based on the interaction with Fc region, and the 20-mer peptide was identified accordingly. Interaction energies (Van der Waals & Electrostatic) of peptide and IgG (Fc) were compared with the B domain interaction with IgG (Fc), where the crystal structure is available. The energy aspects focused chiefly on the stability of the complex with Fc for different peptides. It is directly evident from the comparative study of interaction energies that interactions for B-domain fragment with Fc is stronger than the interactions of similar fragments from other domains. Thus such theoritical prediction could result in identification of a fragment which is also experimentally active.

We demonstrated the IgG binding property in a dose dependent manner (Fig. 1C). The unique ability of the synthetic 20-mer peptide to bind IgG might provide an alternate, and altogether a novel procedure for the separation of IgG and/or immune complexes. Protein A-columns (PA-Colloidal charcoal, PA-glass beads, PA-Sepharose) have been used in experimental cancer therapy (28) to adsorb blocking immunecomplexes which have been reported to be associated with significant morbidity. Purified PA has been demonstrated by

us to have antitumor property (3, 4). The synthetic peptide is expected to be able to avoid some of the problems, as were incountered during the previous observations with PA containing *S aureus*, heat killed and formaline stabilized (2, 29), purified PA itself (30) or PA columns (28).

In order to ascertain the possible immunostimulating property of the peptide, IFNγ, TNFα, IL1α, IL4, IL6 and IL10 were assayed in sera of mice at different time point, and compared with that of PA. The results of Fig. 2A indicate that the peptide upregulates the production of IFNγ, TNFα, IL1α and downregulates simultaneously the production of IL4, IL6 and IL10 as was observed with PA. It is thus clear from Fig. 2B that the peptide evokes Th1 response as that of PA. Since the peptide induces the production of several cytokines *in vivo* that are known inducers of NO, in the next approach we tested whether the peptide can induce NO production also *in vivo*. It was observed that the peptide induces an increased production of NO as that of PA (Fig. 3). It is already established that PA generates Th1 type response in mice (7) and also shows an increased production of NO *in vivo* (26). These observations suggest that the peptide behaves in a manner similar to that of PA at least in terms of the above mentioned properties of PA. In future, we would explore the possibility of its use as a substitute of PA for its other biological properties *viz* antitumor (3, 4), antifungal (8) and antiparasitic properties (9).

Cell cycle analyses indicate that the dose of this peptide was selected on the basis of precipitation reaction, which was observed to be non toxic, as there was no increase in hypoploidy status of the cells of thymocytes as well as spleenocytes (Fig. 4). There was an appreciable transition from Go/G1 to S and G2/M phase of the cell cycle indicating an increased proliferative response in both the populations. PA has been described as potent B and T cell mitogen (10, 11). In non-Hodgkin's lymphomas, which is characterized by arrested stages in cell differentiation, PA has been shown to stimulate [^{3}H]thymidine incorporation as well as shifting of cell cycle from G0/G1 to S and G2M, indicating induction of cell proliferation (17). It is not certain however if the 20-mer peptide would show the probability of its use as a substitute of PA for such activity.

Thus, the 20-mer peptide induced Th1 type response may induce a cascade of signaling events, ultimately causing immunocyte proliferation and cytokine release as well as NO production. These results allow us to hypothesize that the stimulation of different cells involved in the immune response induced by the 20-mer IgG binding peptide described in this report, might have far reaching impact in immunostimulation of the host where it is required for therapeutic benefit.

ACKNOWLEDGMENTS

We are greatly indebted to Dr. Gaurisankar Sa for computational help. We also acknowledge Mr. Subhamoy, Mr. Tapan Mondal, Mr. Uttam Kumar Ghosh, and Mr. Ranjit Kumar Das for technical help and Mrs. Rina Das for typing the manuscript.

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