SECONDARY IMMUNIZATION INHIBITS DRUG INDUCED APOPTOSIS *IN VIVO*

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ABSTRACT

Immunization with a proper dose of an antigenic stimulus leads to cell proliferation and antibody response of circulating lymphocytes. We have previously observed that Secondary immunized spleenocytes resist ceramide-mediated apoptosis *in vitro*. Our present study is aimed at investigating the *in vivo* effect of immunization on apoptosis. Mice were subjected to either Primary or Secondary dose with Tetanus Toxoid. Unimmunized spleenocytes served as controls. Unimmunized, Primary and Secondary immunized mice were later exposed to chemotherapeutic drugs such as Etoposide/ Methotrexate/ Vincristine to induce apoptosis. Apoptosis was studied by using the Feulgen reaction on 5 μ thin paraffin sections of spleen. It was observed that Secondary immunized mice showed a lower percentage of apoptosis as compared to Primary or Unimmunized mice that was subjected to either of the chemotherapeutic drugs. It was thus concluded that Secondary immunization inhibits the process of chemotherapeutic drug induced apoptosis *in vivo*.

KEY WORDS

Apoptosis, cellular proliferation, T lymphocytes.

INTRODUCTION

Clonal proliferation of T cells as a result of antigenic stimulation gives a biphasic proliferative response (1). At low doses of the antigen, cells respond with an increase in the proliferative response as a function of increasing stimulatory dose (2). It has been seen that more intense lethal dose of the same antigen causes negative selection and cell death (3,4). An optimum dose of the antigen provides a high cell proliferation stimulus, which leads to an antibody response. In recent years, it has been observed that apoptotic death occurs after the end of the antigenic stimulus. This process must be tightly regulated in order to retain sufficient T cell progeny to mediate an effective response, while allowing the rapid deletion of these cells at the end of the

response to prevent lymphadenopathy and cross-reactive immunity. A recent study (5) has investigated, the factors regulating the sensitivity of T cells to apoptosis *in vitro* after the induction of primary T-cell activation within a mixed lymphocyte reaction (MLR). We have previously observed that Secondary immunized spleenocytes resist ceramide-mediated apoptosis *in vitro*. However, the question whether immunization inhibits apoptosis *in vivo* was not addressed so far. Accordingly, the aim of the present study was to investigate the response of Unimmunized, Primary and Secondary immunized spleenocytes to apoptotic induction by chemotherapeutic agents such as Etoposide, Methotrexate or Vincristine.

MATERIALS AND METHODS

Experimental design: Four weeks old swiss albino mice were divided into 2 groups:

(1) Control group (n=18), (2) Apoptosis stimulated groups. In group 2, apoptosis was induced *in vivo* by chemotherapeutic drugs such as Etoposide (n=18), Methotrexate (n=18) and Vincristine (n=18)

Each of the above groups (Control, Etoposide,

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Methotrexate and Vincristine) was further divided into 3 subgroups each such as Unimmunized (n=6), Primary immunized (n=6) and Secondary immunized subgroups (n=6).

Immunization – Mice in the Primary immunized group were immunized with a single dose of 0.1 ml Tetanus toxoid (30 µg, Serum Institute of India, India). Mice in the Secondary immunized group received the first dose of Tetanus toxoid followed by 4 booster doses at 2 weeks interval (6,7). Antibody levels in this group were quantitated by Single Radial Immunodiffusion method and Double Diffusion method (8,9).

Apoptosis induction - Apoptosis was induced in the Unimmunized, Primary and Secondary immunized mice by Etoposide [(75 mg/kg body surface area (BSA), Dabur India Ltd, India) (10,11)], Methotrexate [(25 mg/kg BSA, Neon Antibiotics Ltd, India) (12,13)] or Vincristine [(0.5 mg/kg BSA, Biochem Pharm Industries, India) (14,15)]. These chemotherapeutic agents were injected intraperitoneally for 5 days commencing from 5 days after the last antigenic dose or equivalent age in the unimmunized group.

TISSUE PROCESSING FOR DETECTION OF APOPTOSIS:

Fixation– Mice were anaesthetized with anaesthetic ether and sacrificed 48 hours after the last dose of the chemotherapeutic agent or equivalent age in the Control group. The spleen was then removed and fixed in Bouin's fluid for a minimum of 48 hours.

Paraffin sectioning- Tissue blocks were processed for paraffin sections using routine histological procedures (16). 5µ thick sections were taken and mounted on the gelatinized slides.

Detection of apoptosis- As reported earlier, apoptosis was detected in histological sections by using the Feulgen reaction (17,18). The sections were dewaxed with xylene and hydrated with descending grades of ethyl alcohol and placed in distilled water for 5 minutes. The sections were placed in Schiff's reagent for 45 minutes. It was then rinsed in bisulphite solution (10% potassium metabisulphite) followed by wash in distilled water. Counter staining of the sections were done with 1% light green. The slides were dehydrated with ascending grades of alcohol, cleared with xylene and finally mounted with DPX mounting media. The

slides were observed under light microscope at 400 X magnification for detection of apoptotic cells which stain brick red in colour.

Quantification of Apoptotic cells - Percentage of apoptotic cells/1000 µ² area was counted using a calibrated ocular grid at 400 x magnification. Slides were coded in order to avoid experimenter's bias. From each spleen a minimum of 5 sections were selected for quantification. The minimum distance between the two adjacent sections selected for quantification was 15 µ, in order to avoid the duplication of counting in the adjacent sections. In each section, 10 randomly selected fields of 1000 µ² was selected for quantification. Mean percentage of apoptotic cells/1000 µ² area was thus calculated for each mice spleen.

RESULTS

- 1) Serum antibody level- secondary immunized mice showed sufficient immunization as quantitated by the Single Radial Immunodiffusion (SRID). The serum antibody level was 1.7 mg %.
- 2) Apoptosis in Control groups– The sections of spleen from control groups (which have not received chemotherapeutic drugs) showed a lower percentage of apoptotic spleenocytes in the secondary immunized subgroups as compared to unimmunized and primary immunized subgroups (Fig. 1).
- 3) Apoptosis in the chemotherapeutic drug treated groups- In all the chemotherapeutic drug treated groups, secondary immunized subgroups showed a low percentage of apoptotic spleenocytes as compared to Unimmunized and Primary immunized subgroups (Fig. 2, 3, 4).
- 4) Comparison of the control and chemotherapeutic drug treated groups- Analysis of a 5µ thick section has revealed that the apoptotic groups containing secondary immunized spleenocytes respectively show a lower number of apoptotic cells as compared to the control group (Table 1).

DISCUSSION

Primary Immunized spleenocytes have shown a higher percentage of apoptosis due to a short

immune response. Chemotherapeutic drugs induce T cell apoptosis in spleenocytes (19). The mode of action of these drugs on spleenocytes is already well established (20,21). *In vitro* analysis has revealed that secondary immunized spleenocytes are more resistant to ceramide mediated apoptosis as compared to unimmunized or primary immunized spleenocytes (communicated) (3). In this study, analysis of the percentage of apoptotic cells/1000 μ^2 area in the control groups have revealed a significant decrease in the number of apoptotic cells in the secondary immunized subgroups as compared to unimmunized and primary immunized subgroups [(Table 1) (ANOVA, Bonferroni's test)]. Similar results have been observed in the apoptotic groups wherein secondary immunization shows a highly significant decrease in the number of apoptotic spleenocytes as compared to that of the unimmunized and primary immunized subgroups ($p < 0.0001$).

Histopathological analysis of the splenic tissue (5 μ thickness) showed a difference in the number of apoptotic cells between the control and chemotherapeutic drug mediated groups. However, this result showed a considerable variation when enumerating the number of apoptotic cells of the entire splenic tissue having a length of 2 cms [(4000 sections)(1mm=1000 μ)].

In vitro flowcytometry analysis has also revealed a lower percentage of apoptotic spleenocytes in the secondary immunized cells as compared to the primary and unimmunized cells in both the control and apoptotic groups analyzed (Fig 5). The comparison of the control and the apoptotic groups were shown (Table 2). The fluorescence spectra were stored as histogram plot in the list mode and were later analyzed using the Cell Quest software for data analysis.

The mechanism of inhibition of apoptosis may be

the same as explained earlier (5). During Secondary stimulation it was found that activated T cells rapidly acquire the expression of both Fas and Fas ligand (FasL) on their surface and contain a high level of the precursor form of the proapoptotic enzymes caspase-8 (FLICE). Secondary immunization, which results in a higher antibody production, causes a simultaneous increase in the expression of FLICE inhibitory protein (FLIP) and the inhibition of the expression of the apoptotic gene FLICE. However, T cells were resistant for up to 5 days to apoptosis following stimulation of Fas; a maximal apoptotic response was observed after 7 days. This coincided with a marked reduction in expression of FLIP and maximal activity of caspase 8. At time periods beyond day 7, the number of viable cells decreased, as the expression of Interleukin-2 (IL-2) at this period was low, hence resulting in a decrease in expression of the antiapoptotic protein bcl-2. Apoptosis was thus seen, as there is a leakage of cytochrome c from the mitochondria resulting in direct activation of the caspase cascade.

Feulgen reaction provides an accurate assessment of apoptotic cells. During apoptosis, chromatin condensation takes place (19) which results in a free -OH group at the 3' end of the deoxyribose sugar of the condensed DNA. The exposed aldehyde at the 3' end of the deoxyribose sugar binds to Schiff's reagent staining it brick red in colour.

As primary and unimmunized mice show an increased percentage of apoptotic spleenocytes when administered with chemotherapeutic drugs as compared to secondary immunized spleenocytes, it clearly showed that secondary immunization inhibits the process of apoptosis.

ACKNOWLEDGEMENTS

This work was done with the grant from the Indian Council of Medical Research (ICMR).

Fig. 1. Photomicrographs of spleen from Unimmunized (A), Primary Immunized (B) and Secondary immunized (C) mice (treated without any chemotherapeutic drugs). Note the increased number of apoptotic cells (arrows) in (A) and (B) as compared to the Secondary immunized subgroup (C). (Scale bar- 20 μ , Feulgen reaction).

Fig. 2. Photomicrographs of spleen from Unimmunized (A), Primary immunized (B) and Secondary immunized (C) mice (treated with Etoposide drug). Note the Increased number of apoptotic cells (arrows) in (A) and (B) as compared to the Secondary immunized subgroup (C). (Scale bar- 20 μ , Feulgen reaction).

Fig. 3. Photomicrographs of spleen from Unimmunized (A), Primary immunized (B) and Secondary immunized (C) mice (treated with Methotrexate drug). Note the increased number of apoptotic cells (arrows) in (A) and (B) as compared to the Secondary immunized subgroup (C). (Scale bar- 20 μ , Feulgen reaction).

Fig.4. Photomicrographs of spleen from Unimmunized (A), Primary Immunized (B) and Secondary Immunized (C) mice (treated with Vincristine drug). Note the increased number of apoptotic cells (arrows) in (A) and (B) as compared to the Secondary immunized subgroup (C). (Scale bar- 20 μ , Feulgen reaction).

FIGURE 5

Fig 5. Flowcytometry analysis of Unimmunized, Primary and Secondary immunized spleenocytes from the Control and apoptotic groups. Control groups consist of a) Unimmunized b) Primary immunized and c) Secondary immunized subgroups. Apoptotic groups consist of d) Unimmunized e) Primary immunized and f) Secondary immunized subgroups. Histogram plots were analyzed by using the Cell Quest software for data analysis.

Table 1
Percentage of Apoptotic cells/unit area

Type	n	Control	Etoposide	Methotaxate	Vincristine
Unimmunized mice (UI)	6	14.28	50	43.33	59.48
Primary Immunized mice *# (PI)	6	70.37	78.80	79.86	72.18
Secondary Immunized mice \$ +(SI)	6	20.38	20	10.15	15.94
A					
N F		49.798	34.353	67.103	81.808
O P		<0.0001	<0.0001	<0.0001	<0.0001
V					
A					

Percentage of apoptotic cells / 1000sq micron area of splenic section. Each value represents the mean of 10 random fields (1000 μ^2 area) / section (5 sections / mice, n=6 / group). Each value represents Mean Percentage value. UI v/s PI: * p<0.0001; # p<0.05 (Vincristine group), UI v/s SI: + p<0.0001; UI v/s SI: \$- NS (Not significant-Control group). One way ANOVA, Bonferroni's test.

Table 2
Flowcytometry analysis of different groups
(Percentage of apoptotic spleenocytes in each group)

TYPE	Control	Apoptotic groups
a) UNIMMUNIZED	3.34%	27.31%
b) PRIMARY IMMUNIZED	2.18%	35.78%
c) SECONDARY IMMUNIZED	1.17%	18.14%

Flowcytometry analysis of the Control and apoptotic groups. Each value represents Mean percentage of apoptotic cells in the control and apoptotic groups analyzed. Note the decrease in the percentage of apoptotic spleenocytes in the Secondary immunized subgroups of the apoptotic groups as compared to the Unimmunized and Primary immunized subgroups. The Histogram plot is stored in the list mode and later analyzed by the Cell Quest software for data analysis.

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