CHAPTER 8

IN VITRO IMMUNOSUPPRESSIVE STUDY OF CYCLOSPORINE LIPOSOMES

8.1 INTRODUCTION

The possible use of flow cytometric analysis to detect chemical-mediated effects on immunity and immune system status has engendered much discussion. Flow cytometry was considered to be a very powerful tool for measuring the specific position of a cell in the cell cycle or in another cascade of biologic events based on the expression of various intracellular or surface markers. However, the relationship between the expression of activation markers on immune cells and the function of those cells remains to be determined. Currently, phenotypic analysis for immunotoxicity testing is done with spleen cells from mice and to a lesser extent from rats. Although there are a number of reagents available for studies of mice, for most other species the knowledge of and experience with reagents, surface markers, and procedures is limited. This lack of information limits the use of flow cytometric analysis in species other than the mouse. Factors potentially limiting the use of primates for immunotoxicity testing include the use of peripheral blood as the source of cells for flow cytometric analysis, limited availability of reagents, limited information on most surface markers. To date, most phenotypic evaluations in preclinical immunotoxicity testing have used the mouse spleen. Only limited data are available on the effects of immunotoxicants on peripheral blood leukocytes in the mouse, and in cases where data from both peripheral blood and the spleen are available, changes in peripheral blood phenotypes do not always correspond to effects on spleen cell populations. Sampling of peripheral blood offers a number of advantages: blood can be obtained by a relatively noninvasive method, multiple samples can be collected over time, and findings in the peripheral blood of animals may facilitate extrapolation to humans because human peripheral blood is generally available for evaluation. However, peripheral blood can only provide information on the types and numbers of leukocytes present at the time of sampling and may simply reflect trafficking of immune cells. Moreover, the volume of blood in mice is limited and the types and numbers of leukocytes in the blood may not reflect those found in lymphoid tissue. This is important in the context of immune function because the primary and secondary immune organs are where immune responses are initiated through cell-cell interactions.

Immunophenotyping as part of an immunotoxicity assessment should be done using spleen cells rather than peripheral blood cells when the goal is preclinical hazard identification. Because information on the effects of immunotoxicants on peripheral blood leukocytes is limited, more studies (in various species) are needed to determine whether findings in the periphery correlate with spleen cell data.

Dose dependence is an important feature of a true toxicologic effect, and for many immunotoxicants, lower doses stimulate the immune system whereas higher doses are suppressive. Some known immunosuppressive chemicals have dose-dependent effects on specific types of murine spleen cells: dideoxyadenosine produces a dose dependent decrease in B cells, and cyclosporine A produces dose-dependent alterations in T and B cells. However, dose-response data are limited to a small number of well-studied immunotoxicants. When assessing for dose-dependent effects on immune cell phenotypes, it is important to use a graded series of concentrations of the test compound, starting with a dose that is at or near the maximum tolerated dose. More information is needed to determine whether dose-dependent changes in phenotypic profiles are a common effect of immunotoxic chemicals. A key area for further investigation is the relationship between changes in phenotype or activation status and changes in immune function as an indicator of immune competence. For a limited number of immunotoxicants, dose-dependent changes in the profiles of T cells, B cells, or both have been reported, but the relationship between these changes and immune function have not been confirmed. In other cases no significant correlations have been observed between changes in phenotypic profile and immune function. This limited information suggests that alterations in cell phenotypes or activation status observed during preclinical testing are unlikely to indicate changes in immune function. However, in clinical testing such changes (e.g., alterations in CD4+ T cells) can indicate altered immune function.

Apoptosis is a mechanistically driven form of cell death that is either developmentally regulated or activated in response to specific stimuli or various forms of cell injury. In order to evaluate the relevance of *in vitro* methods for immunosuppressive assessment, the effects of cyclosporine on

the apoptosis of mouse splenocytes were studied. A comparison of apoptosis of immune cells from spleen following an *in vitro* exposure to cyclosporine (CsA) and liposomal cyclosporine has been done. These data show the usefulness of *in vitro* systems for immunosuppressive assessment.

8.2 EXPERIMENTAL

8.2.1 CHEMICALS

Propidium Iodide (PI), fetal calf serum, penicillin, streptomycin, and L-glutamine were purchased from Sigma chemical Co., St.Louis, M.O.; Fluorescein (FITC) labeled anti-rabbit IgG were from Pharmingen, San Diego, USA. All other chemicals and solvents were of analytical reagent grade and were used without further purification.

8.2.2 PREPARATION OF SPLENIC LYMPHOCYTES (SPLENOCYTE) SUSPENSION

The spleen was removed from 8 weeks old mouse weighing about 25g under sterile conditions. Splenocytes were obtained by gently pressing the tissues between sterile glass slides using sterile PBS (pH 7.4). Clumps were removed by filtration through nylon mesh. The splenocytes were processed into single cell suspension and erythrocytes were lysed with Tris buffered ammonium chloride, (0.16 M of ammonium chloride; 0.17 M Tris = 9:1, pH-7.2). The cells were washed again, refiltered and counted by haemocytometer.

8.2.3 TREATMENT OF SPLENOCYTES WITH CSA AND LIPOSOMAL CSA

Splenocytes ($1X10^6$ cells/ml) were either incubated in PD-35 petri plates containing 2ml of RPMI-1640 medium supplemented with 10% fetal calf serum, 4mm L-glutamine, 50 units/ml penicillin and 50 μg /ml streptomycin at 37°C in a 5% CO_2 and 95% air-humidified incubator for several periods (6, 12 and 24h) with different concentration of CsA (0.1 μM, 1.0 μM and 10 μM) or cultured for 24h with two different doses (1.0 μM and 10 μM) of liposomes encapsulated CsA. Untreated splenocytes incubated for the same periods served as controls. Analysis of the nuclear DNA content as well as CD4 and CD8 status were carried out by flow cytometry.

8.2.4 DNA ANALYSIS BY FLOW CYTOMETRY

Apoptosis of CsA treated splenocytes was measured by flow cytometry essentially according to a method reported previously (Nicoletti et al., 1991, Darzynkiewicz et al., 1992). Experiments were carried out with either freshly isolated cells or cells cultured for 24h. Splenocytes were treated with varying concentrations of CsA or liposome encapsulated CsA for 6-24h. The cell suspension from the petri dish was transferred into centrifuge tube and was centrifuged for 10 minutes at 300Xg. Cells were washed once with PBS and suspended in 0.5 ml of PBS (pH 7.4). Fixation was carried out by adding the cell suspension to 1.5ml of chilled absolute alcohol on a vortexer. Fixed cells were stored at 4°C overnight. DNA was stained essentially according to the method described earlier (Dwarakanath et al., 1999). Briefly, ethanol was removed by centrifugation (300Xg), washed once with PBS and treated with 200 µl of RNAse (200 µg/ml) for 30 minutes at 37°C. DNA was stained by adding 300 µl of Propidium Iodide (PI) (50 µg /ml), and the samples were stored at 4-8°C for 1 hr before measurements. Flow cytometric measurements were made using a FACS Calibur flow cytometer (Becton-Dickinson, USA) having a 488nm laser excitation. Data from 10,000 cells was acquired using the "Cell Quest"software and analyzed using the Mod-fit program supplied by the manufacturer.

8.2.5 ANALYSIS OF CD4 AND CD8 CELLS

The immunofluoresence of splenocytes stained with anti-CD4 and CD8 antibodies was measured by flow cytometry according to a method reported previously (Wilkins et al., 2002, Kajioka et al., 2000). Splenocytes ($1X10^6$cells/ml) were incubated for 24h with 1.0 µM of free CsA and liposomes encapsulated CsA at 37°C in a humidified atmosphere containing 5% CO_2. After 24hr incubation cells were washed twice, resuspended in PBS. Approximately $0.5\text{-}1*10^6$ cells were incubated with 200µl rabbit anti-mouse CD4 or CD8 antibody (Pharmingen, USA) for 30 minutes at 4°C and washed twice with PBS. The pellet was dislodged with PBS and incubated with the 200µl of FITC-labeled anti-rabbit IgG. The green fluorescence was measured on a FACS- Calibur flow cytometer (Becton-Dickinson, USA) using the Cell

Quest software as described earlier. Analysis of CD4 and CD8 positive cells was carried out with appropriate off-line gating.

8.2.5 STATISTICAL ANALYSIS

Statistical analysis was performed using Microsoft Excel software (Microsoft Office XP, Microsoft Corporation, U.S). Results are expressed as mean ± standard error of mean for three experiments. The student t' test was carried out to test the presence of statistically significant difference at the level of $P<0.05$.

Figure 8.1 Flow cytometric DNA profiles of PI stained splenocytes treated with different concentrations of cyclosporine *in vitro* along with the percentage apoptosis of splenocytes shown below each DNA histograms

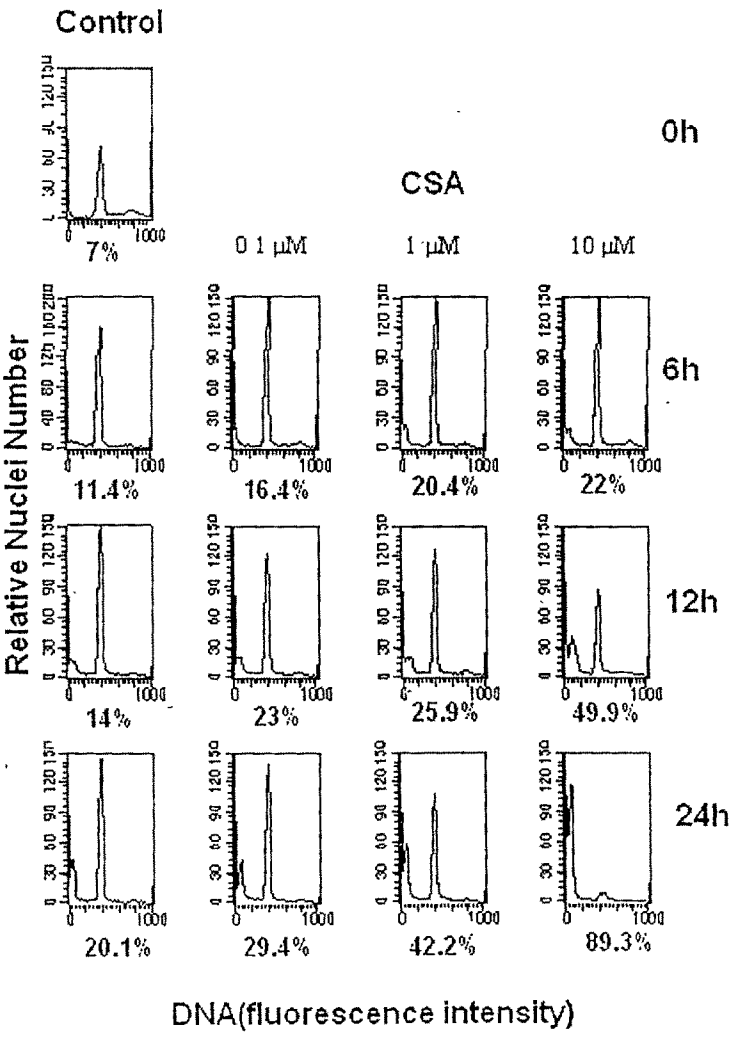
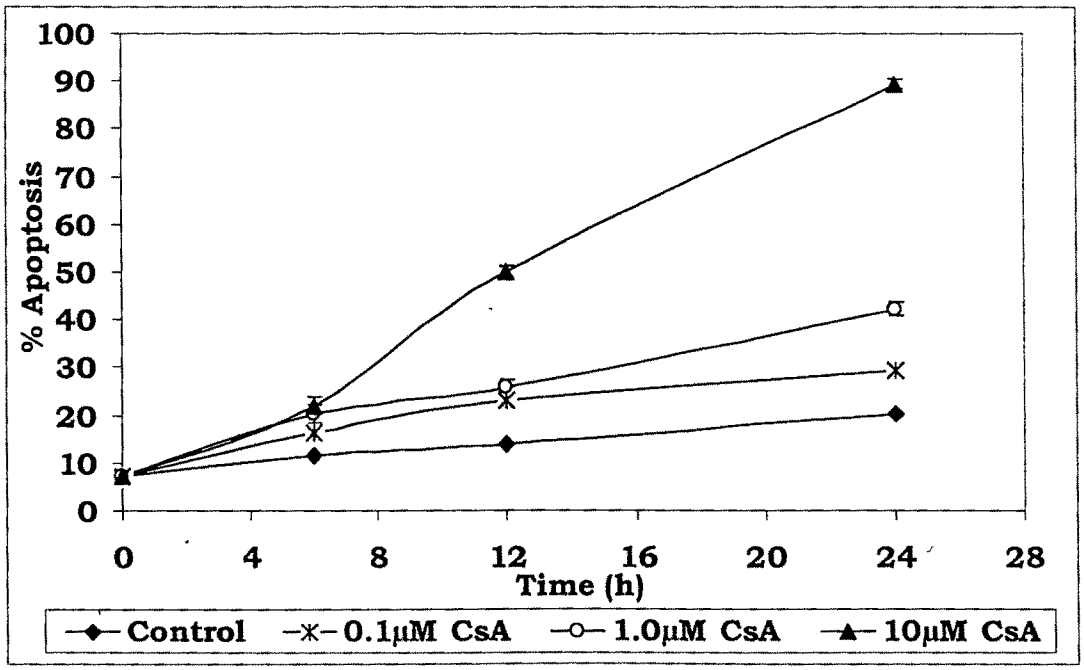


Table 8.1 Concentration dependent apoptosis of cyclosporine on mouse splenocytes

Time (h)	% Apoptosis			
	Control	0.1μM CsA	1.0μM CsA	10μM CsA
0h	7±1.02	7±1.02	7±1.02	7±1.02
6h	11.4±1.23	16.4±1.05	20.4±2.01	22±1.65
12h	14.0±1.42	23.0±0.99	25.9±1.65	49.9±1.44
24h	20.1±1.22	29.4±1.32	42.2±1.36	89.3±0.99

Figure 8.2 Concentration dependent apoptosis of cyclosporine on mouse splenocytes



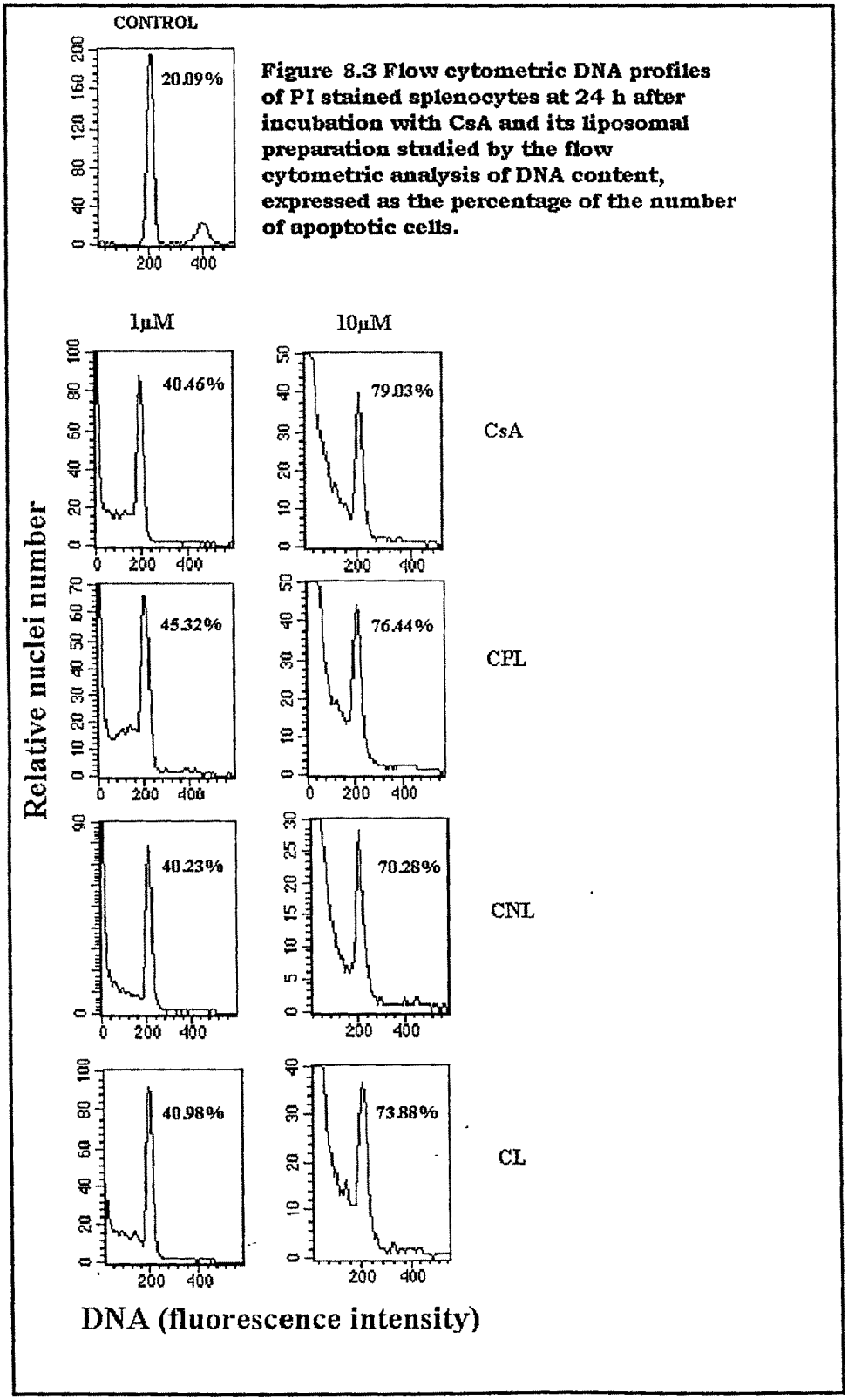


Figure 8.3 Flow cytometric DNA profiles of PI stained splenocytes at 24 h after incubation with CsA and its liposomal preparation studied by the flow cytometric analysis of DNA content, expressed as the percentage of the number of apoptotic cells.

Table 8.2 Induction of apoptosis in Splenic lymphocytes at 24 h after incubation with CsA and its liposomal preparation studied by the flow cytometric analysis of DNA content, expressed as the percentage of the number of apoptotic cells

Samples	Concentration of cyclosporine	
	1μM	10μM
Control	20.09±1.02	20.09±1.06
Drug	40.46±1.23	79.03±1.36
CPL	45.32±1.05	76.44±1.45
CNL	40.23±2.05	70.28±0.92
CL	42.36±1.23	73.88±2.25

Figure 8.4 Induction of apoptosis in Splenic lymphocytes at 24 h after incubation with CsA and its liposomal preparation studied by the flow cytometric analysis of DNA content, expressed as the percentage of the number of apoptotic cells. Values represent mean ± S.E.M from three independent experiments

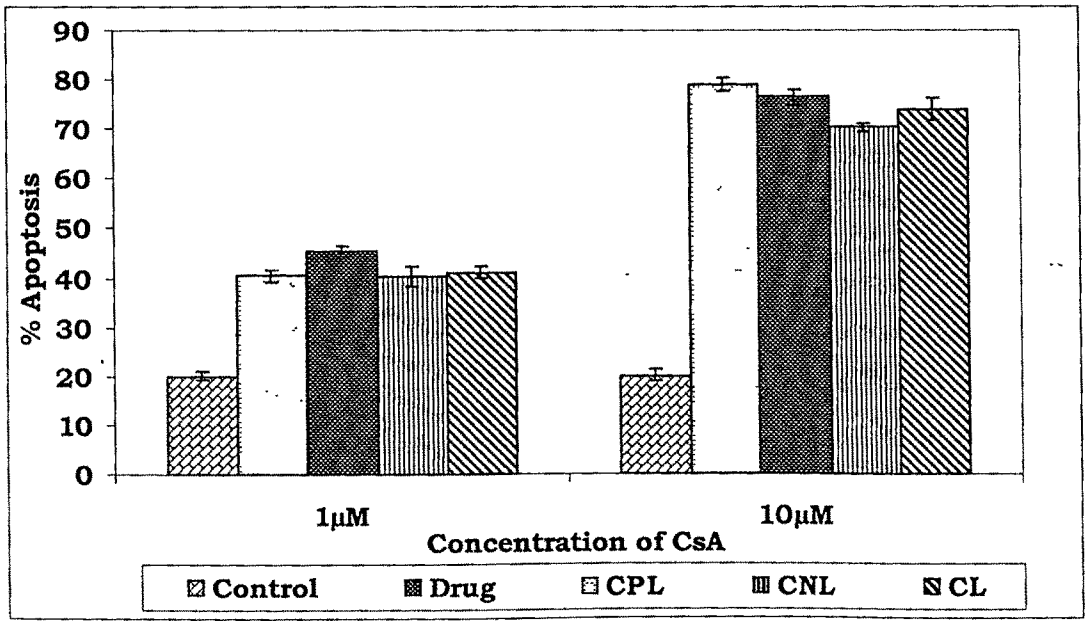


Table 8.3 Percentage of CD4+ cells and CD8+ cells in the spleen treated with 1.0μM of CsA and liposomal CsA

	% of CD4+ cells	% of CD8+ cells	Relative number
Control	42.3	36.9	1.15
Drug	24.9	27.0	0.92
CPL	24.6	26.7	0.92
CNL	22.8	25.6	0.89
CL	21.5	24.7	0.87

8.3 RESULTS AND DISCUSSION

The *in vitro* immunosuppressive study was carried out as a preliminary study before carryout the *in vivo* biodistribution study of the liposomes encapsulated immunosuppressive drug (cyclosporine). This was carried out to evaluate the potential of the immunosuppressive drug when encapsulated in charged liposomes.

8.3.1. STUDIES ON APOPTOSIS BY FLOW CYTOMETRY

The present study addressed the *in vitro* activity of CsA and its liposomes by analyzing apoptosis related DNA degradation using flow cytometric determination of DNA content in cultured splenocytes. Apoptosis is a mechanistically driven form of cell death that is either developmentally regulated or activated in response to specific stimuli or various forms of cell injury. There are three criteria for judging apoptosis: morphology of cells, detection of DNA fragmentation and sub diploid DNA content measured by flow cytometry. To ensure the reliable quantitation of the apoptotic activity of CsA, the fraction of apoptotic splenocytes was gauged by measuring the DNA content of nuclei stained with PI (Propidium iodide) by flow cytometry according to the procedures already described (Nicoletti et al., 1991). Untreated splenocytes cultured for the same periods also displayed apoptotic characteristics, suggesting that the untreated splenocytes underwent spontaneous apoptosis. However, the fraction of apoptotic nuclei was significantly lower than that of the treated splenocytes. The induction of apoptosis by CsA was found to be time and CsA concentration dependent

(figure 8.2). Since changes in the concentration of drug could alter the kinetics of cell death induced by drug, we studied the kinetics of apoptosis at different drug concentration. The DNA analysis showed that the percentage of apoptotic nuclei (hypo diploid) in the CsA treated splenocytes increased with the incubation time (6, 12 and 24h) and CsA dose. Figure 8.1 shows the DNA histogram of untreated splenocytes and splenocytes treated with different concentration of CsA for 6, 12 and 24h and the table 8.1, figure 8.2 showing the concentration dependent apoptosis of cyclosporine at various time intervals. Table 8.2, figure 8.3 shows the DNA histogram of untreated splenocytes and splenocytes treated with different concentration of CsA and liposomal CsA for 24h and figure 8.4 shows the comparison of the prepared liposomal formulations (CPL, CNL and CL) containing two different concentrations (1.0μM and 10μM) of CsA with free CsA on the induction of apoptosis. CPL induced a marginally higher level of apoptosis at 1.0μM as compared to free CsA, whereas CNL and CL exhibited similar as free CsA at both these concentrations, suggesting the efficiency of liposome mediated drug delivery.

8.3.2 EFFECTS ON CD4 AND CD8 CELLS

Incubation of splenocytes obtained from the four weeks old mice with 1.0μM of free CsA and its liposomal preparation induced a decrease in the levels of both CD4 and CD8 subsets as shown in Table 8.3. The fraction of CD4+ cells decreased from 42% to 25%, while the CD8+ cells dropped from 36% to 27%. However, the ratio of CD4 to CD8 cells did not significantly change under these conditions. Significant differences could not be observed between the effects of liposomal preparation and the free CsA on CD4 as well as CD8 cells.

8.3.3 CONCLUSION

In vitro effects on the induction of apoptosis on splenocytes indicates that CPL at 1.0μM, induces a marginally higher level of cell death than free CsA, while the effects of CNL and CL are similar to free CsA, suggesting the improvement in the efficiency of liposome mediated drug delivery containing CsA. Significant differences could not be observed in the effects of free CsA and the liposomal preparation on the CD4 and CD8 *in vitro*.

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