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Effect of Dehydration and Rehydration of the pH-Sensitive Liposomes Containing Chimeric gag-V3 Virus Like Particle on Their Long-term Stability

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One of the practical limitations with the use of liposomes for delivery of the pharmaceutical substances such as antigens is that liposomes are relatively unstable in storage. In order to extend the stability of liposome in storage without affecting their functional activity, solution-type liposomes were dehydrated to form a structurally intact dry liposomes. Comparative immunological evaluation was carried out for both dry and solution-type liposomes containing gag-V3 chimera, consequently it was found that dry liposomes elicited both humoral and cellular response as efficiently as solution-type liposomes did against the same gag-V3 antigen. Especially, long-term stability of the liposomes was remarkably enhanced by the dehydration made to liposomes without a significant change in its ability to elicit immune response *in vivo*. These results indicate that dry pH-sensitive liposome may become an effective delivery and adjuvant system for general vaccine development.

Key words: dry pH-sensitive liposomes, stability, neutralizing antibody, cytotoxic T lymphocytes, adjuvant

INTRODUCTION

Humoral immunity (virus specific neutralizing antibody) appears to be particularly effective in protecting the spread of cell-free virus in vivo, while not so much effective in protecting the primary viral spread by virus infected cells. For this reason, humoral immunity apparently fails to prevent the progression to AIDS despite the presence of a high level of circulating neutralizing antibodies in nearly all infected seropositive individuals [1-3]. On the other hand, cellular immunity may play a significant role for the protection of the disease progression to AIDS. Therefore, recent attempts to develop effective vaccine against AIDS have focused on the generation of humoral and cellular immunity against HIV-1 antigens.

Conventional vaccines such as killed and subunit vaccines have had difficulties in inducing the humoral and cellular immunity at the same time. Proper carrier (adjuvant) system may be critical to induce both immunity, and many attempts have been reported to be successful employing formulation of exogenous antigen into liposomes [4-9], ISCOM [10-11] and Squalene/tween based oil emulsion [12-13]. Among these adjuvant systems, liposomes were widely used for improving the immunogenicity to protein and peptide antigens. Liposomes as antigen carriers and adjuvants to promote immune response to weak antigens or nonimmunogenic antigens have been well documented. Especially, pH-sensitive liposomes have been used to introduce exogenous antigen to the class I restricted presentation pathway. Thus, antigen-entrapped pH sensitive liposomes could elicit the cellular immune response to protein and peptide antigen. In our previous study, we have reported that chimeric gag-V3 virus like particle entrapped in pH-sensitive liposomes composed of phosphatidylethanolamine-β-oleoyl-γ-palmitoyl (POPE)/choesteryl hemisuccinate (CHOH) elicited the CTL response [9]. We have also reported that the V3 peptide encapsulated pH-sensitive liposomes elicited the virus neutralizing antibody and virus specific CTL response at the same time [14].

However, solution-type liposomes as drug and antigen delivery system are unstable, having relatively short shelf life in general. Therefore, their use in practical applications as an adjuvant for vaccine is seriously limited. Other disadvantage of solution-type liposomes is that it is not stable enough for a long period of storage required for drug and vaccine adjuvant systems. In order to overcome these limitations, we have prepared dry liposomes and thereby, succeeded in improving *in vitro* stability of solution-type liposomes on the long period of storage. In this study, we have compared the adjuvant effect of solution-type and dry liposomes and investigated any change of immunogenicity induced by dry liposomes after long period of storage. We will address the improvement on long-term stability of pH-sensitive liposomes by realizing dry liposome.

MATERIALS AND METHODS

Antigens

The HIV-1 V3 loop peptide (HIV-1 IIIB 315-329, RIQRGPGRAFVTIGK, Mogam, Kyounggi-Do, Korea)

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was synthesized by Fmoc chemistry on an ABI 431 A peptide synthesizer, and purified by preparative high-performance liquid chromatography (HPLC). Purity of peptides was confirmed by analytical HPLC and amino acid analysis. The purification of chimeric gag-V3 virus like particle was carried out as described previously [9].

Preparation of Solution and Dry Type pH-sensitive Liposomes

Multilamellar vesicles (MLV) were prepared by vortex mixing. Lipids (POPE/CHOH = 7 : 3 mole ratio, Sigma, St. Louis, MO, U.S.A.) were dissolved in 1 mL chloroform and dry under a stream of nitrogen gas to form a thin film on the wall of a glass tube. A PBS buffer solution containing chimeric gag-V3 virus like particle antigen was introduced into the glass vial and vortexed vigorously, and phospholipid to protein weight ratio was kept to twenty. Freezing-thawing vesicles (FTV) were prepared as described elsewhere [15]. Dry liposomes were prepared by the same protocol but their hydration was made using PBS containing four equivalence of trehalose and chimeric gag-V3 particle. This liposome was lyophilized in the liposome solution after freezing thawing process. Dry liposomes were rehydrated with distilled water before use. Fluorescent dye encapsulated liposomes containing 8-aminonaphthalene 1,3,6-trisulfonic acid disodium salt (ANTS)/p-xylene bis (pyridinium) bromide (DPX) (ANTS and DPX, respectively: Molecular Probe, Eugene, OR) were prepared by the same protocol of making solution-type and dry liposomes. For the measurement of the leakage and pH-sensitivity of liposomes, vesicles were separated from unencapsulated ANTS/DPX by passing through a Sephadex G-50 column (1×20 cm) [16]. Phospholipid concentration was determined as described by elsewhere [17].

Immunization

Female Balb/C (Charles River, Charleston, SC, U.S.A.) were 5~6 weeks of age at the time of the initial immunization and each group of ten mice was immunized with liposomal antigen. 100 μL liposome containing 25 μg of chimeric gag-V3 particle antigen was injected by an intramuscularly (i.m.). Boosting was performed by the same route and dose at day 7 after the first immunization.

ELISA

Sera from immunized mice were tested for anti-V3 peptide activity and anti-gag response using the V3 loop peptides and chimeric particle as coating antigens, respectively. Antibody response against chimeric particle encapsulated pH-sensitive liposomes were determined by a solid-phase enzyme-linked immunosorbent assay (ELISA). Briefly, 96 well plates were coated with 100 mg of chimeric particle and 250 ng peptide per well by incubating at 4℃ overnight. After the plates were washed three times with PBS containing 0.5% tween 20, the wells were blocked with PBS containing 1% BSA at 37℃ for 1 hr. After washing three more times, the plates incubated at 37℃ for 1 hr with serial twofold dilution of the various antisera. Subsequently, they were washed extensively with PBS containing 0.02% tween 20. HRPO-conjugated anti-mouse IgG was reacted with each well for 1 hr at 37℃. After washing, 100 μL of substrate solution (2,2′-azino bis(3-ethyl benzthiazoline-6-sulfonic acid, ABTS) and H_2O_2 were added to each well. Optical Density readings (405 nm) were measured at the 15 min end points using a Biotek reader (SLT, Grodig, Austria).

In vitro Neutralization Assay

The anti-HIV-1 virus neutralization titres of sera from immunized mice were determined by the syncytial forming reduction assay using MT-2 cells. Serial two-fold dilutions of the serum samples were made to make 30 μL of a final volume in flat bottom 96 well microtiter plates in triplicate. An aliquot of the viral stock was five-fold diluted in culture media and 30 μL of the diluted viral stock was added to each well. The plates were then incubated for 1 hr (37℃, 5% CO_2), and after the incubation, MT-2 cells were added to each well in 60 μL aliquots containing 1×10^4 cells. Plates were incubated at 37℃, 5% CO_2 in humid atmosphere for 3~5 days and cytopathic effect including syncytial formation was observed by light microscopy everyday. The neutralization titres were expressed as the reciprocal of the highest serum dilution at which more than 50% inhibition of syncytial formation was observed.

CTL Assay

The spleens were removed from immunized mice 2 weeks after the final immunization, and spleen cell suspensions were prepared and pooled from two mice per group. Splenocytes obtained from immunized Balb/c mice were used for CTL assay. Splenocytes were restimulated *in vitro* for 7 days with V3 loop peptides derived from IIIB strain of HIV-1. For the preparation of peptide loaded target cells, p815 [a MHC-matched ($H\text{-}2^d$) mastocytoma cell line], cells (1×10^6/mL) were incubated with V3 loop peptide for 1 hr and labeled with ^{51}Cr for 1 hr at 37℃. The cells were then washed three times with RPMI 1640 medium containing 10% fetal calf serum. Control target cells were incubated with culture medium alone. The CTL activity against peptide loaded target cells were determined in a standard ^{51}Cr release assay. Varying aliquots of effector cells (5×10^5, 2×10^5, and 1×10^5) were mixed with 2×10^4 of ^{51}Cr-labeled, peptide loaded P815 target cells per well in 96-well microtiter plates. Then 100 μL of supernatants from each well was collected for ^{51}Cr release assay. Specific lysis was determined by the following equation: Specific lysis = (Sample release-Spontaneous release)/Maximum release-Spontaneous release) × 100. Spontaneous release was determined for target cells incubated with culture medium and maximum release was determined for target cell lysed by 2% Triton X-100.

To test for effector phenotype, $CD4^+$ or $CD8^+$ cell depleted populations were obtained by incubating effector cells with monoclonal anti-CD4 antibody or anti-CD8 antibody (anti-L3T4 antibody or anti-Ly2 antibody, Boehringer Manheim, Manheim, Germany), respectively [9,14]. Resulting cells were then tested for their activity to lyse MHC-match p815 cells that were pretreated with V3 peptides. Untreated, $CD4^+$ or $CD8^+$ cell depleted populations were incubated at various

Table 1. pH-dependent leakage of liposomes contents with different type of liposomes

Type	pH						
	8.0	7.0	6.5	6.0	5.5	5.0	4.2
Solution	0[a]	0	5	17	35	67	78
Dry	0	0	3	12	27	70	82

a: % leakage
The composition of solution-type and dry liposomes was POPE/CHOH (7 : 3, mole ratio) without or with trehalose, respectively. The pH sensitivity of liposomes was evaluated by the ANTS/DPX method. For the measurement of pH-sensitivity of liposomes at buffer with varied pH values, the liposomes suspension was incubated with buffer with varied pH values at 37℃ for 1 hr and fluorescent intensity was measured. The 0% leakage was defined as the fluorescence intensity measured from intact liposomes containing ANTS/DPX at initial time. The 100% leakage was defined as the fluorescence intensity measured the complete destruction of liposomes with Trition X-100.

effector : target (E : T) ratio of 25 : 1, 10 : 1, and 5 : 1. The culture supernatant of ^{51}Cr labeled cells harvested and determined by ^{51}Cr release assay.

RESULTS AND DISCUSSION

Characterization of Dry pH-sensitive Liposomes

Property of dry pH-sensitive liposomes used in this study was characterized by the pH-sensitivity and leakage of the inner substance on long period of storage conditions. In order to determine the pH-sensitivity of dry liposomes, liposomes were rehydrated with distilled water and mixed with HEPES buffer with different pH values. Solution-type and dry liposomes were stable at neutral pH but the leakage of the inner substance, the leakage increased appreciably as the pH was lowered (Table 1). As shown in Table 1, pH-sensitivity was not altered by dehydration. The pH-sensitivity observed here shows similar leakage range which found by other pH-sensitive liposomes used for inducing CTL activity of exogenous antigen [18].

Stability of solution-type and dry liposomes was performed by leakage assay on long period of storage conditions. Entrapped ANTS/DPX leaked out much more slowly from dry liposomes than from solution type liposomes at 4℃ and 37℃ (Table 2). These results clearly demonstrated that dry liposomes were more stable than the solution liposomes regardless of temperatures. For the case of dry liposomes, leakage did not occur during storage but some leakage (below 5%) was detected at rehydration step with distilled water.

We further examined the change of liposome size from dry liposomes. After lyophilization, the size of dry liposomes did not change in the presence of trehalose but liposome size was dramatically increased in the absence of trehalose. Fig. 1 showed the size distribution of solution and dry liposomes. As shown in Fig.1, dry liposomes in the presence of trehalose showed no significant change in the apparent liposome diameter on long period of storage; it revealed a liposomes population having a mean diameter of $1,400 \pm 300$ nm. But, dry liposome in the absence of trehalose had a mean diameter of $2,500 \pm 400$ nm. Small size shown in Fig. 1 revealed free virus like particle unentrapped into the liposomes. Previously we reported that free chimeric gag-V3 virus like particle had a mean diameter of 110-130 nm by transmission electron microscopy using uranyl acetate staining [9].

Table 2. Stability of solution-type and dry liposomes *in vitro* buffer system at 4℃ and 37℃

Time (month)	Leakage at 4℃ (%)		Leakage at 37℃ (%)	
	Solution	Dry	Solution	Dry
0	0	0	0	0
1	1	3	20	4
3	3	3	82	5
6	5	4	95	5
12	10	5	94	5

The leakage experiment was initiated by adding concentrated liposomes suspensions into magnetically stirred cuvette containing the PBS buffer (pH 7.4) to obtain the final concentration of 30 μM. Fluorescent intensity was measured at 20℃ for 10 min. The 0% leakage was defined as the fluorescence intensity measured from intact liposomes containing ANTS/DPX at initial time. The 100% leakage was defined as the fluorescence intensity measured the complete destruction of liposomes with Trition X-100.

Antibody Response Induced by Chimeric Particle Entrapped Solution-type and Dry Liposome

To test the adjuvant effect of dry pH-sensitive liposomes, we have studied the immune response of chimeric gag-V3 virus like particle encapsulated pH-sensitive liposomes. Each group of ten mice was immunized two times at 7 days interval with liposomal antigen. Dry liposomes were immunized after slightly vortexing on rehydration with distilled water. The immune sera, obtained 2 weeks after the last boost immunization, were tested for their ability to recognize gag protein and V3 peptide from chimeric gag-V3 particle by ELISA. All mice immunized with liposomes generated a strong anti-gag and anti-V3 response (Table 3), demonstrating that the chimeric gag-V3 virus like particle is a potent immunogen and dry liposomes are a good adjuvant system.

The neutralizing response elicited against the IIIB strain by the sera from immunized and control mice was evaluated by the syncytial forming reduction assay. Sera immunized by solution-type and dry liposome adjuvant showed a high neutralizing activity against the homologous strain of HIV-1, while the sera obtained from control mice did not show any neutralizing activity. Moreover, adjuvant effects by solution-type and dry liposomes had no difference in inducing the humoral immune response.

Induction of V3 Peptide Specific CTL Response with Chimeric gag-V3 Particle

Chimeric gag-V3 particle represents three tandem copies of V3 sequence from IIIB strain of HIV-1. To confirm whether this chimeric particle induce V3 peptide specific CTL response *in vivo*, the Balb/C mice were immunized with solution-type and dry liposomes containing 25 μg of purified chimeric gag-V3 virus like particle. Spleens were removed from immunized mice at 2 weeks after final immunization. Splenocytes were restimulated *in vitro* with V3 loop peptide and subsequently used for CTL assay as an effector cells. The resulting effector cells were tested for their ability to lyse the syngeneic target cells preincubated with V3 loop peptide of IIIB strain of HIV-1. As expected, the

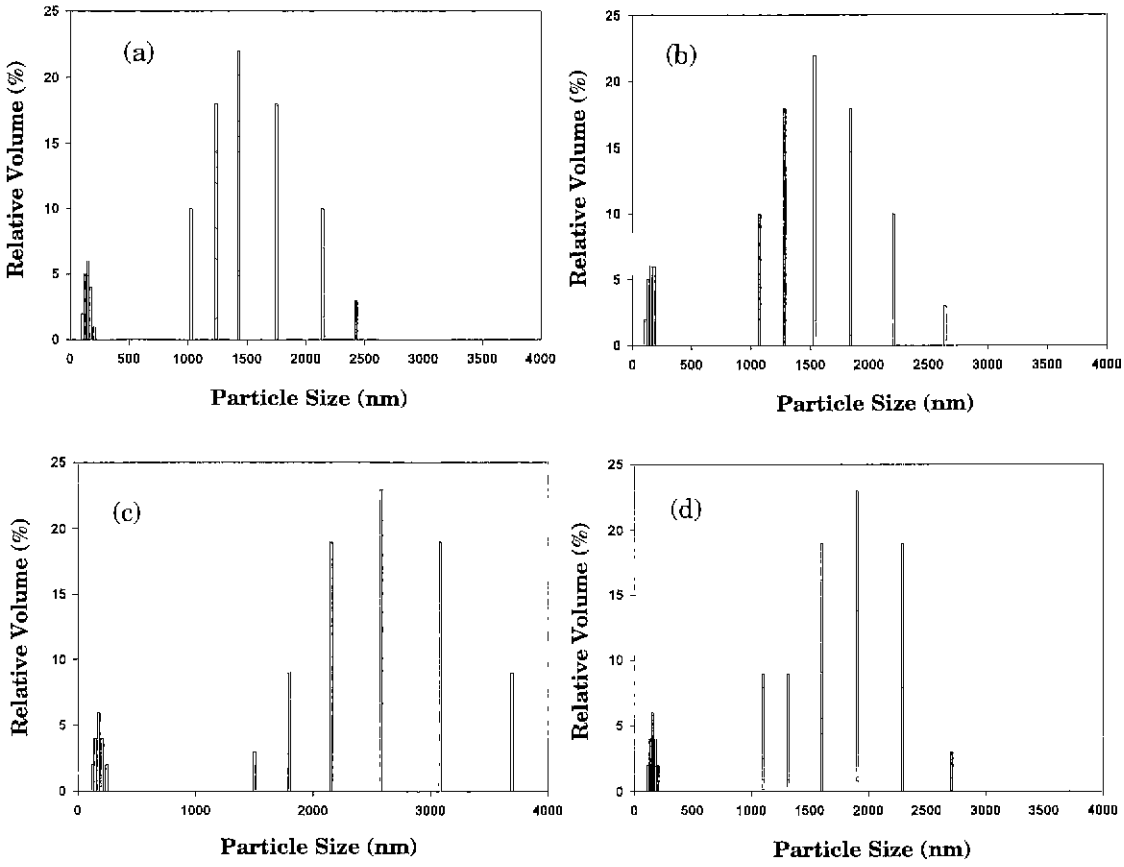

Fig. 1. Particle size distribution of solution and dry liposomes obtained by laser light scattering method at 20℃. (a) solution-type liposomes composed of POPE/CHOH (7 : 3, mole ratio), (b) dry liposomes in the presence of trehalose, (c) dry liposomes in the absence of trehalose, and (d) the change of dry liposomes size in 12 month after storage at 37℃. Size experiment was performed by dilution of concentrated liposomes suspension into cuvette containing PBS buffer (pH 7.4) to obtain the final phospholipid concentration of 50 μM.

Table 3. Antibody and neutralizing antibody titers following two immunization with solution and dry liposomes

Adjuvant	ELISA titer		Neutralizing titer
	V3 peptide coating	Protein coating	
Solution-type	800	8,000-16,000	1,024
Dry	800	16,000	1,291

splenocytes from mice immunized with chimeric gag-V3 particle resulted in specific response for V3 peptide loaded target cells (Fig. 2). As shown in Fig. 2, similar CTL responses were induced at solution-type and dry liposomes. This result is consistent with a previous report that V3 peptide is a strong inducer of CTL [14]. In contrast, V3 peptide loaded target cells were not lysed by effector cells primed by albumin encapsulated pH-sensitive liposomes and ovalbumin peptide loaded target cells were not lysed by the effector cells primed by chimeric gag-V3 particle, either (data not shown). These results indicated that the chimeric gag-V3 particle is capable of inducing V3 peptide specific CTL response *in vivo* and adjuvant effects by solution-type and dry liposomes have no difference in inducing the cellular immune response.

To investigate the stability of dry liposomes on the induction of peptide specific CTL response *in vivo*, we have tested the CTL response of dry liposomes on long period of storage from 0 to 18 months. Dry liposomes

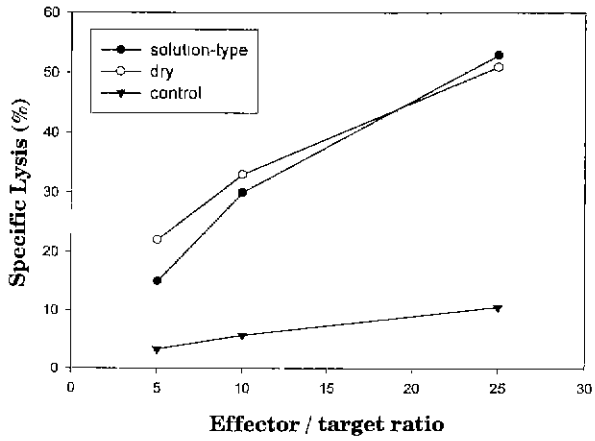

Fig. 2. CTL induction with chimeric gag-V3 virus like particle encapsulated solution-type and dry liposomes. Balb/c mice were immunized with liposomes as described in Materials and Methods. Splenocytes obtained 2 weeks after final immunization were tested for cytolytic activity following *in vitro* culture with V3 peptide. CTL activity was measured using MHC matched target cells that were pretreated with medium alone (p815) or the V3 peptide at various effector to target cells ratio of 5 : 1, 10 : 1, and 25 : 1.

with indicated storage time interval (0, 1, 2, 3, 6, 12, 15, and 18 months) were immunized and splenocytes were tested for cytolytic activity following *in vitro* culture with a V3 loop peptide. CTL generated by solution-type liposomes have significantly decreased

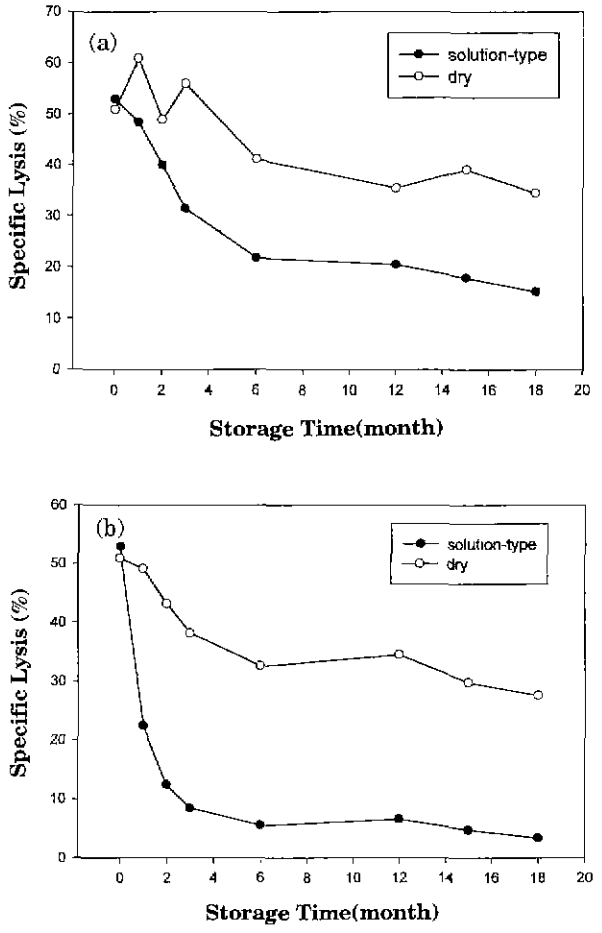

Fig. 3. The change of adjuvant effect to solution-type and dry liposomes on the long period of storage conditions from 0 to 18 months at 4℃ (a) and 37℃ (b). Balb/c mice were immunized with solution-type and dry liposomes at individual time interval. Splenocytes obtained 2 weeks after final immunization were tested for cytolytic activity following in vitro culture with V3 peptide. CTL activity was measured using MHC matched target cells that were pretreated with medium alone (p815) or the V3 peptide at various effector to target cells ratio of 5 : 1, 10 : 1, and 25 : 1.

the levels of CTL activity after one month of storage. In contrast, CTL generated by the dry liposomes maintained a significantly high level of activity for 12 and 18 months after storage at 4℃ and 37℃, respectively (Fig. 3). These results demonstrated that dry liposome was more stable and had higher CTL inducibility than solution-type liposomes.

To investigate whether the CTL function in splenocytes from immunized mice was due to $CD8^+$ or $CD4^+$ T cells, effector CTL were incubated with anti-CD4 or anti-CD8 antibody without complement. Depletion of $CD4^+$ or $CD8^+$ T cells from the restimulated splenocytes by treatment with respective monoclonal antibody revealed that the CTL activity was mainly due to $CD8^+$ T cells (Fig. 4). Therefore, depletion of $CD8^+$ T cells dramatically decreased the cytotoxicity against V3 loaded p815 target cells. In contrast, pretreatment of effect cells with anti-$CD4^+$ antibody had no significant effect. Therefore, it was ascertain that $CD4^+$ T cells were involved in the lysis of target cells somewhat but $CD4^+$ T cells were not necessary for the target cell lysis. These results indicated that the CTL response was mediated by the class I restricted pathway.

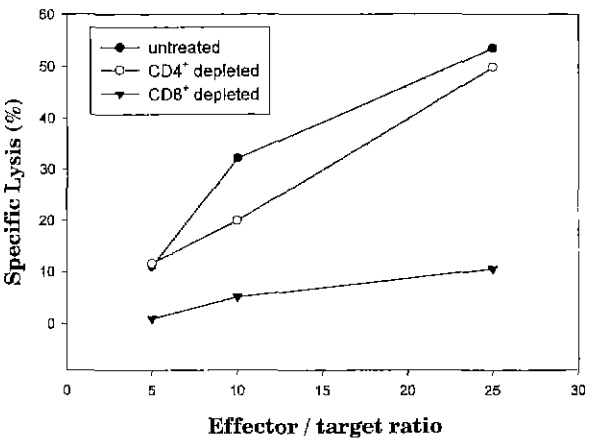

Fig. 4. CTL response induced from immunized mice. Effector cells were incubated with anti-$CD4^+$ antibody and anti-$CD8^+$ antibody as described in the Materials and Methods Untreated, $CD4^+$ T cells depleted, or $CD8^+$ T cells depleted populations were cultured with ^{51}Cr labeled, V3peptide loaded target cells at various effector to target cells ratio of 5 : 1, 10 : 1 and 25 : 1, and ^{51}Cr released was measured.

In conclusion, we have successfully produced the dry pH-sensitive liposomes to improve the stability of solution-type liposomes and investigated any change of immunogenicity on long period of storage. Dry liposomes could be capable of generating humoral (virus specific neutralizing antibody) and cellular (virus specific CTL) immune response as shown to solution-type liposomes. We have found that the adjuvant effect of both type liposomes had no difference in eliciting the humoral and cellular immune response. Our results also indicated that antibody produced by chimeric gag-V3 particle seemed to neutralize HIV-1 and CTL generated by the same antigen was restricted to V3 peptide. Based on the results of this study, dry pH-sensitive liposomes may be used as a practical vehicle for development of various subunit and peptide vaccine. In addition, this liposome is sufficient to use as a drug delivery system.

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