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Postimmunization Clearance of Liposome Entrapped Adenovirus Type 5 Hexon[1] (41321)

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Abstract. Clearance rates of ^{125}I-hexon administered in three different forms from injection sites were assessed in animals. Aqueous and liposome-entrapped hexons following parental administration were cleared in linear fashion with half-lives that ranged from 1.5 to 4 and 30 to 45 hr, respectively. In contrast, hexons emulsified in complete Freund's adjuvant were cleared in a biphasic manner and tended to remain localized with 23 to 55% persisting after 16 days. No significant antigen accumulation in blood or any internal organs was detected following the three modes of immunization. These data suggest that formation of a transient antigen "depot" at injection sites may contribute to the adjuvant effect of liposomes but the complete resolution of this "depot" may avoid significant local reactogenicity.

Adenovirus type 5 hexons and fibers are weak immunogens in unprimed children (Kasel, Drake, and Taber, unpublished observations) and seronegative animals (1, 2). Recent studies in a rabbit model have demonstrated that the relative frequency of serum-neutralizing responses and the level of antibody titers obtained with these proteins entrapped within multilamellar liposomes are comparable to equivalent doses emulsified in complete Freund's adjuvant (CFA) and both vaccine forms are markedly more immunogenic than the aqueous antigens (1). Moreover, hexons and fibers carried in liposomes apparently retain their T-cell-dependent nature, and induce high levels of specific serum antibody of the IgG class (3).

The present studies utilized ^{125}I-labeled hexons to assess the *in vivo* fate of antigens carried in liposomes. Hexons entrapped in liposomes were cleared more slowly than aqueous antigen but in contrast to antigen in CFA, did not persist at the injection site. No accumulation of antigen in regional lymphnodes or any of the internal organs was detected following injection of the three vaccine forms. The results suggest that soluble antigens encapsulated in liposomes were cleared in a manner that may avoid the deleterious reactions associated with emulsion adjuvants.

Materials and Methods. *Purification of adenovirus type 5 hexons.* The hexon protein was purified from soluble antigen extracts of adenovirus infected cells essentially as described by Pereira *et al.* (4).

Radiolabeling of hexons. The purified protein was iodinated (^{125}I) by the chloramine T procedure (5) with minor modifications (6). Radiolabeled hexon was separated from free iodine by molecular sieve chromatography on Sephadex G-200; hexon-containing fractions were pooled, supplemented with 20% heat-inactivated horse serum and 10^{-3} *M* dithiothreitol, and stored at −70°. The specific activity of preparations used in the present study ranged from 10 to 15 μCi of ^{125}I per microgram of protein. Labeled hexons were rechromatographed on 10-ml column of Sephadex G-200 prior to use in antigen clearance evaluations. Columns were developed in sterile pyrogen free saline (Elkins–Sinn, Inc., Cherry Hill, N.J.). Greater than 95% of the radioactivity was precipitable by 10% trichloroacetic acid and greater than 90% by homologous antiserum.

Preparation of multilamellar liposomes. Lipids were purchased from Sigma Chemical Corporation, St. Louis, Missouri, and

[1] A portion of this material was presented at the 1979 meeting of the Interscience Conference on Antimicrobial Agents and Chemotherapy held in Boston, Mass.

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stock solutions were prepared in chloroform or mixtures of chloroform and methanol and stored at −20°. Large multilamellar liposomes were prepared from a mixture of dipalmitoylphosphatidylcholine, cholesterol, and phosphatidic acid in the molar ratio of 2.0:1.5:0.2, respectively (7). The dried lipid film was dispersed by vortexing in sterile pyrogen free saline. For protein entrapment, the solution contained 100 μg of hexon per milliliter and 10^7 to 10^8 cpm of ^{125}I-hexon. Untrapped antigen was removed by centrifugation and the final liposome preparations were resuspended in sterile pyrogen-free saline. Liposome concentrations were determined by total phosphate (8) and trapped antigen was determined by the fluorescamine assay (9) or by radioactivity (1). Trapped hexon ranged from 0.5 to 0.7 μg of protein per micromole of liposomal phospholipid.

Hexon clearance from injection sites. Groups of seronegative white New Zealand rabbits (Rich-Glo Corporation, El Campo, Tex.) or female (C3H/HeJ) mice (M.A. Bioproducts; Walkersville, Md.) were given 1−2 μg of hexon (1×10^5 to 5×10^6 cpm of ^{125}I-hexon) in saline, entrapped in liposomes or emulsified in CFA (Difco Laboratories, Detroit, Mich.). The formation of a stable emulsion was confirmed by placing droplets on water (10). Animals administered entrapped antigen received between 1.3 and 1.9 μmol of liposomal phospholipid. A volume of 0.1 ml of each vaccine form was given either intramuscularly in the calf of the rear leg or intradermally on the back after removal of the hair. Immediately after injection, and after various time intervals at least two rabbits or three mice were sacrificed, the injection sites excised, and the amount of remaining antigen determined by γ-ray counting. In mice, a 0.1-ml sample of blood was collected at each time point (0, 1, 3, 6, 12, 24, and 72 hr for aqueous antigen and 0, 0.5, 1, 3, 5, and 16 days for the liposome and CFA forms). Inquinal lymphnodes, spleens, kidneys, livers, hearts, lungs, and thymuses were also collected, washed, and the amounts of ^{125}I determined.

Radioimmunoprecipitation (RIP) assays. Serum antibody levels to adenovirus type 5 hexon antigen were determined in RIP assays as previously described (3, 6). Each assay tube contained equal 50-μl volumes of: an antisera dilution (fourfold serial dilutions starting at 1:200), 10% normal horse serum, and 0.5% mouse carrier serum. After addition of 50 μl of ^{125}I-labeled antigen (10,000−12,000 cpm) the tubes were incubated at 37° for 3 hr; 50 μl of goat antimouse IgG (Meloy Laboratories, Inc., Springfield, Va.) was added and the tubes were incubated at 37° for 1 hr and then at 4° overnight. Phosphate-buffered saline (1.0 ml) was added and immune complexes were separated by centrifugation. The dilution of antiserum that precipitated 20% of the antigen was determined and antibody titers are expressed as the reciprocal of this dilution.

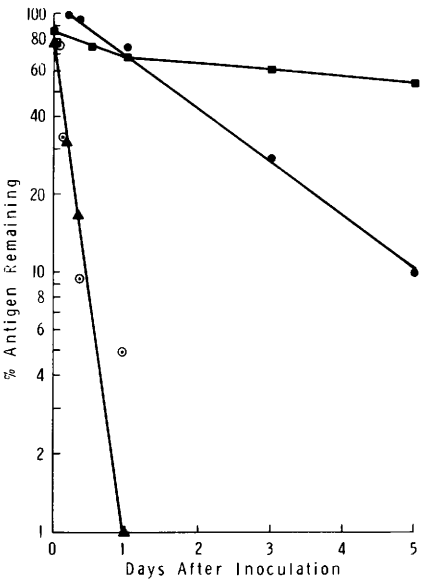

FIG. 1. Effect of vaccine form on clearance rates of ^{125}I-hexon from intradermal injection sites. Seronegative rabbits received 1 μg of hexon (1×10^5 to 5×10^6 cpm) in 0.1 ml of saline (▲), saline mixed with liposomes (⊙), entrapped in liposomes (●) (1.6 mol of phospholipid), or emulsified in complete Freund's adjuvant (CFA) (■). Immediately after injection and after the indicated time intervals animals were sacrificed, injection sites excised, and residual ^{125}I-hexon determined. Each data point represents the mean of four injection sites.

Results. *Antigen clearance from injection sites.* Hexons trace labeled with ^{125}I were utilized to assess persistence of antigen given in different forms at injection

TABLE I. CLEARANCE RATES OF ^{125}I-HEXONS GIVEN IN DIFFERENT FORMS FROM INTRADERMAL AND INTRAMUSCULAR INJECTION SITES[a]

Animal Species	Route of administration	Antigen half-life (in hr) for clearance of hexons injected as indicated		
		Aqueous	Entrapped in liposomes	Emulsified in CFA
Rabbit	Intradermal	4.0	38	>120 (55)[b]
Rabbit		2.8	45	ND[c]
Rabbit	Intramuscular	2.0	30	>120 (23)
Rabbit		2.5	44	>120 (42)
Mouse	Intramuscular	3.2	40	ND
Mouse		2.0	31	>120 (40)
Mouse		1.5	44	>120 (55)

[a] These were computed from semilogarythmic plots of the percentage antigen remaining at the injection site at the different time intervals stated under Materials and Methods. Half-lives indicated for CFA refer to second phase of antigen clearance.

[b] Numbers in parentheses indicate the percentage of antigen remaining at injection sites at 16 days.

[c] Not done.

sites in rabbits (Fig. 1). Hexons in saline were cleared in a rapid linear fashion from intradermal injection sites with a half-life of approximately 4 hr. Entrapment in liposomes drastically reduced the rate of antigen removal; the half-life increased to 38 hr. There was no change in rate of clearance over the 5-day period suggesting that the antigen would ultimately be cleared and this was confirmed in other experiments (i.e., no detectable ^{125}I after 16 days). Internal incorporation was a requirement since aqueous hexons added to liposomes containing saline were cleared at the same rate as that shown for the saline curve. As previously observed with other antigens (12), hexons emulsified in CFA were cleared in a biphasic manner. More than 60% of the antigen was retained at the injection site after 5 days and this percentage was only slightly reduced by 16 days.

The above results and those obtained in other evaluations are summarized in a tabular form in Table I. In a second experiment evaluating antigen retention in rabbits after an intradermal injection of hexons in aqueous or liposome preparations, the relative rates of clearance closely resembled those observed previously. Similar clearance patterns were observed when the three vaccine forms were given intramuscularly to rabbits and mice. While some variation in the rates was observed, the aqueous and liposome-entrapped hexons exhibited linear rates of removal in all five experiments. A portion of the antigen administered in CFA always remained at the injection site but the relative amount appeared to be a property of each emulsion and varied between 23 and 60% on Day 5 in different experiments. Similar results were obtained when antigen was given in incomplete Freund's adjuvant.

In vivo distribution of hexons. Mice administered the three vaccine preparations of hexon by the intramuscular route were examined at periodic intervals for localization of antigen, as stated under Materials and Methods. Following dissemination of hexon from the injection site, low levels of hexon were detectable in the blood. For the aqueous form, the maximum concentration occurred 3 hr postimmunization and it represented 9.3% of the total dose per milliliter of blood. By 24 hr, it declined to <0.5%. Assays for detection of hexon in inquinal lymphnodes, spleens, kidneys, livers, hearts, lungs, and thymuses revealed that the level of hexon at these sites never exceeded 0.5% of the administered dose. When hexons were given in liposomes or CFA, the antigen level in blood was maximal between 12 and 24 hr after injection. However, they never exceeded 1%/ml and, in fact, were decreased at later time points. The difference in levels of circulating antigen between these and that observed with aqueous hexon probably reflect a

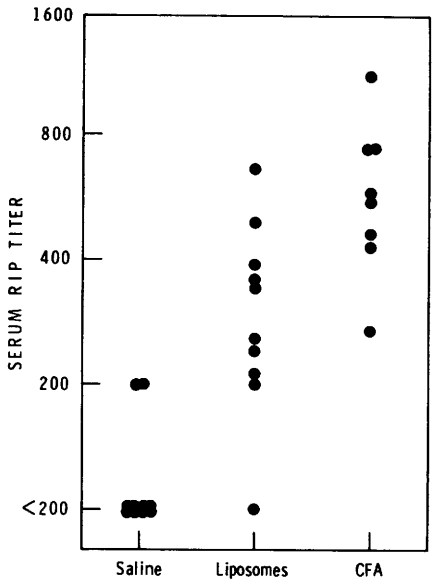

FIG. 2. Serum IgG antibody responses in C3H/HeJ mice following intramuscular inoculation of 5 μg of adenovirus type 5 hexon in different vaccine forms. Serum specimens were obtained from bloods collected by retroorbital puncture before and 4 weeks after vaccination. Antibody levels were determined by RIP assays using ^{125}I-hexon as the test antigen and goat anti-mouse IgG as the second antibody (6). Antibody titers are expressed as the reciprocal of the serum dilution that precipitated 20% of the ^{125}I-hexon. As a control for possible infections by an indigenous mouse adenovirus, two groups of mice were given saline or liposomes without hexon and none of the 20 mice developed a serum antibody response. Inoculation of 1 μg of hexon in aqueous, liposomal, and CFA forms induced serological responses in 0 of 10, 5 of 10, and 8 of 10 mice, respectively (data not shown).

slower dispersion from injection sites. Examination of lymphnodes and all internal organs revealed no accumulation of iodinated antigen (<0.5% of the total dose at all time points). Since previous studies with a variety of antigens have shown that these substances are eliminated from the body primarily by excretion in the urine (11), it was assumed that hexons were removed in a similar manner.

Immunogenicity of hexon in mice. The immunizing capacity of adenovirus type 5 hexon administered in different forms was assessed in seronegative mice and the results are presented in Fig. 2. Hexon given in saline was relatively inefficient at evoking a serum IgG antibody response (RIP titer of ≥200) and these were of low titer. However, entrapment in liposomes substantially increased the number of responding animals (90% vs 20%) and the resulting antibody titers reached higher levels. While the frequency of rises and the range of antibody titers were slightly higher among animals that received hexon in CFA than observed with the liposome form the immunogenicity of these two preparations was comparable. These results are similar to those previously obtained in rabbits (1, 3).

Discussion. The data presented in this report demonstrates that encapsulation in liposomes promotes the retention of soluble antigens at injection sites. However, comparison of the rates that antigen was removed from liposomal and CFA injection sites revealed two important differences in these antigen "depots." Liposome-entrapped hexons were cleared in linear fashion and were completely removed from intradermal and intramuscular injection sites within 16 days. Whereas hexon in the present study, and as previously described by others (12), numerous other antigens in CFA are removed in a biphasic manner, and relatively large amounts persist over several months. Previous studies have shown that persistence of antigen is primarily a function of the oil used in the preparation of the emulsion (12). These differences may simply reflect the fact that liposomes are biodegradable whereas the mineral oil used in CFA is not.

The mechanisms involved in the removal of liposome entrapped antigen from injection sites have not been defined. However, histological examination of intradermal injection sites 24 to 72 hr after administration of liposomes revealed a moderate cellular infiltrate composed predominantly of macrophages (Kramp, Six, Levy, and Kasel, unpublished observations). The cells were highly vacuolated and many of the vesicles contained multilamellar structures that appeared to be liposomes. Similar results have been reported previously and in addition they have suggested that liposomes were generally engulfed intact and subsequently disrupted in the phagosomes (13–15). In view of the known role of macrophages in initiating immune responses

(16) the sequestering of antigen in this type of cell by liposome may also be an important aspect of their adjuvant activity.

Tracing the liposome-entrapped antigen after dissemination from the injection site did not reveal any significant accumulation in lung, spleen, or liver. Following intravenous inoculation entrapped proteins accumulate in these organs (17). Our failure to confirm transient sequestering of antigen in regional lymphnodes following immunization with aqueous and CFA forms (16, 18) may reflect the low antigen dose and later sampling times employed in the present study or the removal of the iodine label from hexons during macrophage processing.

The present study has also shown that liposomes can enhance the immunogenicity of hexons in C3H/HeJ mice. Since mice of this strain do not respond to the adjuvant effects of lipopolysaccharides (19), this result indicates that immunopotentiating activity of liposomes is not due to trace contaminants in the lipid preparations. Moreover, the antibody titers presented in this report may represent an underestimate of the adjuvant effect of liposomes because comparisons of vaccine groups were made with serum specimens collected 4 weeks after immunization. Recent studies indicated that maximum serum antibody levels were attained 8 to 12 weeks after immunization with encapsulated hexon (Kramp, Six, and Kasel, unpublished observations) whereas, in an aqueous solution, the antibody titers usually peak around 4 weeks.

The mechanisms by which adjuvants enhance immune responses are complex and in most instances poorly understood (12, 18). One characteristic of a number of potent adjuvants is the formation of antigen depots at the injection sites. While the depot effect does not fully explain immunopotentiation by these adjuvants, the importance of slow antigen release for inducing maximum serum antibody responses has been established (20). However, it was also observed that persistence of antigen and nondegradable oil for prolonged periods of time (several months) led to granuloma formation and necrosis at the injection site. The complete removal of antigen carried by liposomes may avoid these undesirable side effects.

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