Enhancement of In Vivo Adenovirus-Mediated Gene Transfer and Expression by Prior Depletion of Tissue Macrophages in the Target Organ

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Based on the hypothesis that tissue macrophages present an obstacle for adenovirus (Ad) vector-mediated gene transfer to internal organs, this study evaluated the consequences of transient depletion of Kupffer cells on subsequent transfer of the Ad vector genome and Ad vector-directed gene expression in the livers of experimental animals. Depletion of Kupffer cells in mice by intravenous administration of multilamellar liposomes containing dichloromethylene-bisphosphonate permitted subsequent intravenous administration of an Ad vector to provide a higher input of recombinant adenoviral DNA to the liver, an absolute increase in transgene expression, and a delayed clearance of the vector DNA and transgene expression. These observations suggest that the tissue macrophages pose a significant hurdle to Ad vector-mediated gene transfer and that strategies to transiently suppress macrophage defenses might be useful in enhancing the efficiency of this in vivo gene transfer system.

Replication-deficient, recombinant adenovirus (Ad) vectors are remarkably efficient at transferring genes to target cells in vivo and at expressing the exogenous genes at a high level (8, 12, 28, 29, 54). However, the usefulness of these vectors for potential therapeutic applications is limited by a variety of host defenses arrayed against viruses (22, 44, 49, 55, 56). Based on the observations that in vivo Ad vector-mediated gene expression can be extended in time in a variety of immunodeficient animals (3, 5, 14, 47, 48, 53, 57), various strategies to suppress the host immune response with the goal of making the vector "stealth" to the immune system (1, 8, 18, 50), or to suppress the ability of the cellular immune system to eliminate the target cells that have been infected with the Ad vector (5, 15, 51-53, 57), have been evaluated.

In all of these strategies to suppress host responses in favor of the vector, the focus has been on the adaptive (i.e., antigen-specific) immune system (1, 3, 5, 14, 15, 18, 22, 44, 47-53, 55-57). In contrast, the central hypothesis of the present study is that by suppressing the macrophage component of the innate (i.e., non-antigen-specific) immune system in host defenses against viruses, the Ad vector gene transfer should be enhanced in extent and/or chronicity. We have focused on the macrophage arm of the innate immune system, thus theoretically suppressing the ability of the host to interfere with the extent of Ad vector-mediated gene transfer in vivo and the subsequent extent and chronicity of expression of the newly transferred gene. To accomplish this, we have capitalized on the knowledge that the intravenous administration of multilamellar liposomes encapsulating dichloromethylene-bisphosphonate (Cl₂MBP-liposomes) transiently suppresses the number of Kupffer cells in liver (4, 10, 11, 17, 24, 26, 33-39, 42, 45) and we have used this transient depletion of tissue macrophages to ask whether subsequent administration of an Ad vector to the liver results in modification of gene transfer and

expression in the target organ. Strikingly, the data demonstrate that pretreatment with Cl₂MBP-liposomes enhances both the extent and persistence of expression of the transgene carried by the Ad vector.

MATERIALS AND METHODS

Kupffer cell depletion. Kupffer cells were depleted in vivo by intravenous administration of Cl₂MBP-liposomes (Cl₂MBP [Clodronate] was a gift of Boehringer, GmbH, Mannheim, Germany) as described by Van Rooijen and colleagues (35, 38). The Cl₂MBP-liposomes were used within 3 days of preparation. Controls included intravenous administration of liposomes alone or Cl₂MBP alone. The depletion of Kupffer cells was verified by immunohistochemical analysis on liver sections of Cl₂MBP-liposomes-treated and untreated (control) animals with the monoclonal antibody F4/80, as described by Austyn and Gordon (2). An isotype-matched antibody for glial fibrillary acidic protein was used as a control (6). Quantification of F4/80-positive cells was determined by two observers not associated with the study who randomly and blindly assessed four high-power fields (approximately 100 to 500 hepatocytes/field) of the F4/80 immunohistochemistry sections per specimen, with a total of five specimens (each from a different mouse) per group.

Ad vector. The recombinant Ad vector AdCMV.CATgD (referred to in this work as AdCMV.CAT) is an Ela⁻, partially E1b⁻, and partially E3⁻ Ad vector based on the Ad5 genome (13). It contains an expression cassette using the cytomegalovirus early promoter/enhancer, an artificial splice sequence, followed by the bacterial chloramphenicol acetyltransferase (CAT) gene and the mouse β^{maj} globin poly(A) stop sequences (13). The vector was propagated and purified, the titer was determined, and the purified vector was stored at -70°C, as previously described (28, 29).

In vivo studies. An inbred strain of C57BL/6 mice was used for most experiments (Charles River Laboratories, Wilmington, Mass.); BALB/c and BALB/c-immunodeficient *nu/nu* animals (Charles River Laboratories) were used for comparison. The animals were either sex, were 3 to 4 weeks old, and weighed 15 to 20 g. For administration of the Cl₂MBP-liposomes, vector, or controls, the mice were anesthetized for 3 min by inhalation with methoxyflurane (Pittman-Moore, Mundelein, Ill.). The Cl₂MBP-liposomes (200 μ l) or phosphate-buffered saline (PBS) (pH 7.4; 200 μ l) was administered via the right jugular vein. Controls included liposome-PBS (200 μ l) or Cl₂MBP (1.5 mg in 200 μ l). Two days later, a period when Kupffer cell depletion was maximal (37), the Ad vector AdCMV.CAT (5×10^6 or 10^8 PFU in 100 μ l of isotonic saline/animal as indicated) was administered via the left external jugular vein. The animals were sacrificed at the time points indicated. After being rinsed in PBS, pH 7.4, the liver of each animal was removed and immediately frozen in liquid nitrogen. The center section of the right lobe was used for CAT expression analysis, and the middle section of the right lobe was used for DNA extraction. During the course of experiments, untreated and treated mice showed no signs of ill health and all animals survived until sacrifice.

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CAT assay. The quantitative analysis for CAT activity was carried out by the method of diffusion into scintillation fluid as described by Neumann et al. (23). Briefly, the frozen center section of the right lobe of the liver was weighed and stored in liquid nitrogen. The samples (100 mg) were homogenized on ice in 400 μ l of homogenizing buffer [25 mM glycine-glycine, 4 mM ethylene glycol-bis(β -aminoethyl ether), 15 mM MgSO_4 , and 1 mM DL-dithiothreitol, pH 7.8]. The homogenate was pelleted at $17,500 \times g$ (15 min, 4°C), and the supernatant was heated at 65°C for 15 min. After repelleting ($17,500 \times g$, 15 min, 4°C), the samples were prepared for determination of radioactive counts as described by Sambrook et al. (31). Each sample was counted in triplicate, until the enzymatic reaction was completed (24 h). To help us transform the radioactive counts into units per milligram of tissue, each set of analyses contained a standard curve of CAT enzyme (Sigma, St. Louis, Mo.) diluted in isotonic saline (1 to 100 U/ μ l) followed by dilution of the CAT enzyme in pooled supernatant of 10 uninfected mouse livers (1 to 100 U/ μ l). The resulting enzyme activity of the samples was expressed as units per milligram of tissue. The sensitivity of the assay was >0.1 U/mg of protein. There were no differences between the liver weights of the study groups ($P > 0.2$). The average protein content was 65 mg per ml of supernatant; there were no significant differences between the groups ($P > 0.2$).

Ad vector genome. Quantification of the amount of Ad vector genome in the liver was carried out by extraction from the liver as described by Miller et al. (21). Briefly, the tissue was digested overnight with proteinase K solution (1 mg of proteinase K in 1% sodium dodecyl sulfate in DNA lysis buffer [10 mM Tris-HCl, pH 7.4, 400 mM NaCl, 2 mM EDTA]). Saturated NaCl (5 mM) was added and the DNA in the supernatant was precipitated with ethanol and washed two times with 70% ethanol. For all preparations, the A_{260} -to- A_{280} ratio was between 1.8 and 1.9. The purified DNA (20 μ g) was digested with *Hind*III and evaluated by Southern analysis using 1% agarose gel electrophoresis, transferred to a nylon membrane (Duralon; Stratagene, La Jolla, Calif.), hybridized with a ^{32}P -labeled 440-bp Ad E4 probe prepared by random priming, and evaluated by autoradiography (31). The sensitivity of the assay was >0.004 phosphorimager unit/20 μ g of DNA, the amount of DNA loaded on each lane of the Southern analysis.

Immunity directed against the Ad vector. To determine if cytotoxic T cells (CTL) were induced by administration of the AdCMV.CAT vector in control and Cl_2MBP -liposome-treated animals, C57BL/6 mice (control and pretreated with Cl_2MBP -liposomes) were administered 5×10^6 PFU of the AdCMV.CAT vector, and 28 days later, cells recovered from the spleen were evaluated for CTL against the vector by standard methods (32, 47). In brief, the spleen cells were incubated in vitro for 5 days with syngenic C57SV fibroblasts (kindly provided by B. Knowles, The Jackson Laboratory, Bar Harbor, Maine) infected with the AdCMV.CAT vector and the expanded cell population was evaluated for CTL against the same targets labeled with ^{51}Cr and infected with AdCMV.CAT. To determine if administration of the AdCMV.CAT vector in control and Cl_2MBP -liposome-treated C57BL/6 mice induced neutralizing antibodies against the Ad vector, serum was evaluated for anti-Ad5 neutralizing antibodies at 28 days after vector administration by methods previously described (19).

Statistical analysis. All data are presented as the mean $\pm$ standard error of the mean. Comparison of the numbers of Kupffer cells in the liver and liver weights was carried out by the two-tailed Student *t* test, and time course analysis of the amounts of DNA and CAT in liver was done by the Mann-Whitney test.

RESULTS

Kupffer cell depletion and Ad vector infection of the liver. As described by Van Rooijen and colleagues (35, 38, 39), intravenous administration of Cl_2MBP -liposomes results in a depletion of Kupffer cells in the liver after 2 days (Fig. 1). The Kupffer cell-depleted mice that received 5×10^6 PFU of AdCMV.CAT intravenously all completed the experiment to the predetermined time points for sacrifice. Blinded quantification of the numbers of Kupffer cells in liver 3 days after Cl_2MBP -liposome administration showed 0.3 ± 0.1 F4/80-positive cells/high-power field compared to 14.3 ± 1.5 F4/80-positive cells/high-power field in the PBS control ($P < 0.0001$).

Consequences of Kupffer cell depletion for Ad-mediated transgene expression. The consequences of Kupffer cell depletion for Ad-mediated gene transfer to the liver were evaluated by measuring transgene CAT activity in the liver 7 days after infection with 5×10^6 PFU of AdCMV.CAT to those in control animals and in those animals that had previously received Cl_2MBP -liposomes. There was no difference in CAT expression 7 days after AdCMV.CAT treatment among mice pretreated 2 days before with PBS, empty liposomes, or Cl_2MBP without liposomes ($P > 0.07$, all comparisons; data not shown). In contrast, CAT expression in the livers of animals pretreated

with Cl_2MBP -liposome was significantly higher than that of controls ($P < 0.001$, all comparisons; not shown).

Evaluation of the time-dependent AdCMV.CAT-directed expression of CAT in the livers of control mice compared to that in Cl_2MBP -liposome-treated mice demonstrated that both the extent and duration of CAT expression were markedly enhanced by Cl_2MBP -liposome pretreatment (Fig. 2A). In this regard, CAT expression was enhanced in the Cl_2MBP -liposome groups at all time points examined from 1 day to 4 weeks ($P < 0.02$, all comparisons). At days 1 to 7, when CAT expression was continuing to increase in both groups, the CAT expression per milligram of liver tissue was 7- to 13-fold greater in the Cl_2MBP -liposome group. At week 2, the CAT expression in the Cl_2MBP -liposome group was 20 U/mg, 10-fold higher than the barely detectable expression in the control group. At week 4, while the CAT activity in the control group was undetectable, the average CAT expression in the Cl_2MBP -liposome group was 38% of the peak value observed (day 7) in the control group (Cl_2MBP -liposome group at 28 days, 11 ± 3 U/mg; control group at 7 days, 29 ± 8 U/mg).

Quantification of the amount of vector genome in the liver was consistent with the observation of CAT gene expression (Fig. 2B). The amount of vector DNA in the Cl_2MBP -liposome group was a minimum of twofold higher than that in the control group at all time points ($P < 0.05$, all comparisons), with the value at week 4 in the Cl_2MBP -liposome group in the same range as that in the control group at 1 week. At week 4, the value for vector DNA in the Cl_2MBP -liposome group was 0.05 U/20 μ g of DNA, a value 10-fold higher than the detectable level. In contrast, the vector DNA in the control group could not be detected at this time point.

To determine if the observed enhancement of CAT expression in the liver by the pretreatment with Cl_2MBP -liposomes was dose dependent, in a parallel experiment, C57BL/6 mice were administered (intravenously) 5×10^6 PFU of AdCMV.CAT (as in Fig. 2) and 10^8 PFU of AdCMV.CAT. CAT expression was evaluated at 7 and 14 days after vector administration. The same enhancement in AdCMV.CAT-directed CAT activity in liver was observed with the 20-fold-increased 10^8 -PFU dose as with the 5×10^6 -PFU dose (CAT activities on day 7 for control and Cl_2MBP -liposome-treated mice receiving the 10^8 -PFU dose, $31,500 \pm 8,800$ and $240,000 \pm 22,000$ U/mg, respectively [an eightfold difference; $P < 0.001$]; respective values on day 14, $11,300 \pm 3,200$ and $74,000 \pm 5,200$ U/mg [a sevenfold difference; $P < 0.001$]).

Evaluation of the amount of CAT expression in the liver in control and Cl_2MBP -liposome-treated BALB/c mice 24 h after intravenous administration of 5×10^6 PFU of AdCMV.CAT demonstrated a 27-fold increase in the amount of CAT expression in the livers of Cl_2MBP -liposome-treated animals ($P < 0.01$). A similar pattern was observed in immunodeficient BALB/c *nu/nu* mice, with an 11-fold increase in the amount of CAT expression in the livers of Cl_2MBP -liposome-treated animals ($P < 0.05$). Thus, macrophage depletion in the liver had similar effects on enhancement of the amount of CAT expression transferred in different strains (C57BL/6, BALB/c) and in immunocompetent and immunodeficient animals (BALB/c, BALB/c *nu/nu*).

Taking the data of quantification of CAT expression in the liver (Fig. 2A) and quantification of the vector genome in the liver (Fig. 2B) and replotting both so that the values of each group were 100% of that group's value at day 1 demonstrated that not only did the Cl_2MBP -liposome pretreatment result in enhanced amounts of vector DNA and CAT expression at day 1 but also that there was enhanced persistence of both the vector DNA and CAT expression over time, even when the

FIG. 1. Consequences of intravenous administration of Cl₂MBP-liposomes on liver Kupfer cells. Shown are the results of immunohistochemical detection with the monoclonal antibody F4/80 of Kupfer cells (arrows) on liver sections of control mice (A) and mice that had received Cl₂MBP-liposomes 3 days previously (B). All samples were stained with 3,3'-diaminobenzidine tetrahydrochloride-hematoxylin. Magnification (both panels), $\times 400$.

data were normalized for the initial values (graph not shown). This was observed for both the CAT expression and DNA expression plots, which diverged over time, with the control values falling more rapidly than the Cl₂MBP-liposome values. This same divergence was observed when the DNA and CAT expression data were graphed with normalizing of the data at the peak values for both (day 7 values).

Interestingly, despite the ability of pretreatment with Cl₂MBP-liposomes to enhance the extent and persistence of vector expression, it did not prevent the development of CTL or neutralizing immunity directed against the vector. In this regard, control C57BL/6 mice evaluated 28 days after AdCMV.CAT administration (5×10^6 PFU, intravenous) demonstrated

CTL against a syngeneic target infected with the same vector (average 57% lysis of target at an effector-to-target ratio of 50 to 1), but CTL were also observed in the Cl₂MBP-liposome-treated mice evaluated in parallel (53% lysis at an effector-to-target ratio of 50 to 1). Likewise, control mice evaluated 28 days after AdCMV.CAT administration exhibited neutralizing antibodies against Ad5 in serum (average titer, 40), but so did the Cl₂MBP-liposome-treated mice (titer, 60; $P > 0.5$).

DISCUSSION

Although Ad vectors are very effective in transferring DNA to cells in vivo, the expression of the transgene progressively

FIG. 2. Time course of CAT transgene expression (A) and the amount of Ad vector genome (B) in the livers of control mice and mice treated with Cl₂MBP-liposomes prior to administration of the AdCMV.CAT vector. The mice received 200 μ l of PBS (○) or Cl₂MBP-liposomes (□) 48 h before intravenous administration of 5×10^6 PFU of AdCMV.CAT. (B) The amount of Ad genome was determined by Southern analysis. For both panels, the data are expressed as means $\pm$ standard errors of the means (error bars) (five mice per group at each time point).

decreases over time (3, 5, 8, 12, 14, 15, 28, 29, 47–53, 57). At least in part, the elimination of the Ad vector genome results from recognition of viral and/or transgene epitopes by the adaptive immune system (3, 5, 8, 14, 22, 44, 47–49, 53, 55–57), and the chronicity of expression of in vivo Ad vector-mediated gene transfer can be extended by elimination of viral genes within the vector or by pharmacologic suppression of the immune system (1, 5, 8, 15, 18, 50–53, 57).

The present study focused on the role of the mononuclear phagocyte system in the host defense against Ads and the consequences of transient elimination of mononuclear phagocytes within an organ on the extent and chronicity of expression of a transgene carried by an Ad vector administered to that organ. Based on the knowledge that intravenous administration of Ad vectors results in >90% transfer of the vector genome to the liver and that intravenous administration of

Cl₂MBP-liposomes to rodents results in transient depletion of the liver mononuclear phagocyte system, the results demonstrate that temporary depletion of tissue macrophages prior to administration of Ad vectors results in a markedly increased amount of vector genome transferred to the liver, an increased extent of expression of the transgene, increased persistence of the Ad genome, and increased persistence of expression of the transgene.

Use of Cl₂MBP-liposomes to assess the function of Kupffer cells in innate immunity. Kupffer cells, situated throughout the liver sinusoids, are the liver representatives of the mononuclear phagocyte system (43). Because of their contact with portal blood, they have access to infectious agents passing through the liver and are believed to play a major role in innate immune defenses against circulating infectious agents (20, 43). One strategy to assess the function of Kupffer cells in host defense is to administer to experimental animals compounds that are phagocytized by the Kupffer cells and subsequently interfere with Kupffer cell function and/or induce their destruction (7, 16, 30, 43). Among these strategies, the most successful is intravenous administration of Cl₂MBP-liposomes (4, 10, 11, 17, 24, 26, 33–38, 42, 45).

Cl₂MBP is a member of the bisphosphonate family, which comprises analogs of pyrophosphate, a naturally occurring molecule in bone (25). When Cl₂MBP is packaged in multilamellar liposomes and then administered intravenously to experimental animals, the liposomes are phagocytized by Kupffer cells and, to a lesser extent, by macrophages in the red pulp of the spleen (37, 38). Lysosomal phospholipases disrupt the phospholipid bilayers of the liposomes, and the Cl₂MBP induces the cells to undergo apoptosis (40). The consequence of intravenous administration of Cl₂MBP-liposomes to rodents is the depletion of Kupffer cells in the liver for 1 to 7 days, until new mononuclear phagocytes can establish residence along the liver sinusoids (37, 41). Treatment with Cl₂MBP-liposomes makes animals more susceptible to a variety of diseases and organisms that are normally suppressed by the innate immune system, including disseminated candidiasis (26), *Plasmodium berghei* (42), *Listeria monocytogenes*, and herpes simplex virus type 2 (24). Finally, treatment with Cl₂MBP-liposomes protects animals from disorders in which macrophages play a role in inflammation- or immunity-mediated pathogenesis, including chronic, relapsing autoimmune encephalitis (11) and decreased endotoxin-induced mortality (34).

Implications for the use of Ad vectors for in vivo gene transfer. The demonstration of an increase in Ad-mediated gene transfer and expression by temporary depletion of tissue macrophages has important implications for Ad-mediated gene transfer in vivo. It suggests that the innate immune system plays a significant role in limiting the efficiency of Ad-mediated gene transfer. This concept is consistent with the observation that the innate immune system is responsible for elimination of approximately 90% of an Ad vector reaching the liver within the first 24 h, a time prior to initiation of adaptive immune mechanisms (46). Interestingly, whereas changes in vector design or pharmacologic suppression of the adaptive immune system result in an increase in the persistence of expression (1, 5, 8, 15, 18, 50–53, 57), the data in the present study suggest that suppression of the macrophage arm of the innate immune system not only extends that chronicity of expression but also enhances the initial (and subsequent) extent of expression. Thus, the innate immune system appears to play a significant role in host defense against Ads, and suppression of the innate immune system increases the efficiency of Ad-mediated gene transfer. In this context, it is possible that this increased efficiency of vector delivery may decrease the amount of vector

available to the mononuclear phagocyte system and thus may decrease the antigenic burden presented to the immune system. However, the innate immune system is complex (9), and it is likely that macrophages play only a partial role in innate immune defenses against Ad vectors. Further, both antivector CTL and neutralizing antibodies were observed in the Cl₂MBP-liposome-treated and the control animals, suggesting that host defenses against Ad vectors, including cellular, humoral, and innate immune mechanisms (such as macrophages), are very complex and that simple suppression of only one arm of antiviral defenses will not be adequate to completely circumvent the array of processes directed toward eliminating the newly transferred Ad genome. With this caveat, the observation in the present study may have important applications for human gene therapy. Both liposomes of the type used in this study (27) and Cl₂MBP have been used intravenously in humans (25), and thus it is feasible that this strategy of transient depletion of tissue macrophages may be applicable to in vivo Ad-mediated gene transfer in the clinical setting.

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