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Different Doses of Adenoviral Vector Expressing IL-12 Enhance or Depress the Immune Response to a Coadministered Antigen: the Role of Nitric Oxide[1]

Juan José Lasarte, Fernando José Corrales, Noelia Casares, Ascensión López-Díaz de Cerio, Cheng Qian, Xiaoming Xie, Francisco Borrás-Cuesta, and Jesús Prieto[2]

Joint immunization with two recombinant adenoviruses, one expressing hepatitis C virus (HCV) core and E1 proteins and another expressing IL-12 (RAdIL-12), strongly potentiates cellular immune response against HCV Ags in BALB/c mice when RAdIL-12 was used at doses of 1×10^5–1×10^7 plaque-forming units. However, cellular immunity against HCV Ags was abolished when higher doses (1×10^8 plaque-forming units) of RAdIL-12 were used. This immunosuppressive effect was associated with marked elevation of IFN-γ and nitric oxide in the serum and increased cell apoptosis in the spleen. Administration of N-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase, to mice that received high doses of RAdIL-12 was lethal, whereas no apparent systemic toxicity by L-NAME was observed in those immunized with lower doses of the adenovirus. Interestingly, in mice immunized with recombinant adenovirus expressing core and E1 proteins of HCV in combination with RAdIL-12 at low doses (1×10^7 plaque-forming units), L-NAME inhibited T cell proliferation and CTL activity in response to HCV Ags and also production of Abs against adenoviral proteins. In conclusion, gene transfer of IL-12 can increase or abolish cell immunity against an Ag depending of the dose of the vector expressing the cytokine. IL-12 stimulates the synthesis of NO which is needed for the immunostimulating effects of IL-12, but apoptosis of T cells and immunosuppression ensues when IFN-γ and NO are generated at very high concentrations. *The Journal of Immunology,* 1999, 162: 5270–5277.

A n abundant body of evidence indicates that immune protection against intracellular pathogens and tumors requires the induction of an effective Th1 type of response (1). Recent studies have shown that IL-12 is the most potent cytokine to direct T cell response toward a Th1 profile stimulating the development of IFN-γ-producing T cells (reviewed in Ref. 2). In addition, IL-12 enhances cytotoxic cell activity and proliferation of activated T and NK cells (3). In animal models of infection with intracellular bacteria, it was found that the pathogen induces the development of a Th1 response by stimulating macrophages to produce IL-12 (4). Thus, this cytokine appears to bridge the gap between innate immune response and the acquisition of specific T cell-mediated immunity.

Because of these reasons, IL-12 has been proposed as a potentially effective adjuvant in prophylactic and therapeutic vaccines (2). According to these expectancies, IL-12 was found to be highly efficient as an adjuvant in different models of infections such as *Leishmania major* (5–7), *Schistosoma mansoni* (8), respiratory syncytial virus, and pseudorabies virus (9, 10). The beneficial effects of IL-12 also extend to tumoral immunity (11–13). However,

it has been shown that under certain circumstances and at high doses, IL-12 inhibits virus-specific cytotoxic T cell activity and is detrimental to resistance against viral infection (14, 15). On the other hand, safety studies in rodents and primates revealed that IL-12 has a rather small therapeutic window (16) and in fact, significant adverse effects followed systemic administration of recombinant IL-12 in human clinical trials designed to evaluate the antitumoral effect of this cytokine (reviewed in Ref. 17).

Although increased circulating IL-12 levels following the injection of the recombinant cytokine can produce untoward side effects, the use of adequate doses of gene transfer vectors expressing IL-12 in association with vectors containing DNA coding for the Ag might allow continuous low level production of the cytokine at the right site facilitating immunostimulation without toxicity (18, 19). In a previous work (20), we found that s.c. injection of a recombinant adenovirus expressing core and E1 proteins of hepatitis C virus (HCV)[3] (RAdCE1) induces a cytotoxic T cell response against a diversity of epitopes from core and against one specific epitope from E1 (peptide E1 121–135). These studies have been conducted to investigate the ability of recombinant adenoviruses to induce cellular immunity to HCV Ags in mice as a previous step to the use of these vectors in primate models of HCV infection and eventually in humans. To explore whether an adenoviral vector expressing IL-12 (RAdIL-12) could enhance cellular immunity to HCV Ags, in the present work we have analyzed T cell responses in animals injected with RAdCE1 alone or in combination with different doses of RAdIL-12. Because nitric oxide has emerged as an important modulator of T cell function (21–26),

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[3] Abbreviations used in this paper: HCV, hepatitis C virus; RAdCE1, recombinant defective adenovirus expressing hepatitis C virus core and E1 proteins; RAdIL-12, recombinant defective adenovirus expressing IL-12; RAdLacZ, recombinant defective adenovirus expressing *LacZ* gene; L-NAME, N-nitro-L-arginine methyl ester; pfu, plaque-forming units.

we have also studied the implication of this mediator in the immunoregulatory effects produced by the different RAdIL-12 doses used as adjuvant. Our results indicate that low doses of RAdIL-12 potentiate T cell immunity against HCV Ags whereas high doses had an inhibitory effect on T cell responses. Nitric oxide was found to play a critical role in the immunomodulatory effects induced by IL-12; low levels of NO were required for IL-12-mediated immunostimulation whereas IL-12-mediated immunosuppression was associated with very high NO production.

Materials and Methods

Generation of recombinant adenoviruses

RAdCE1 under the control of the CMV promoter and recombinant adenovirus containing the reporter gene *LacZ* under the control of the same promoter were generated as previously reported (20). Recombinant adenovirus RAdIL-12 containing the two IL-12 subunits p35 and p40 and expressing functional IL-12 under control of the CMV promoter was constructed as follows: *Hind*III/*Spe*I fragment of pBS/p35 containing cDNA of the p35 subunit of IL-12 was filled in by Klenow and blunt-end ligated into *Bam*HI-cut pMV100 which carries CMV promoter and poly(A) signal. The resulting plasmid was digested by *Hind*III to release p35 expression cassette that was ligated into *Eco*RI-digested pΔE1sp1A by blunt-end ligation (pΔE1sp1A/p40). The *Nco*I/*Sma*I fragment of pBS/p40 containing the p40 subunit of IL-12 was cloned into *Nco*I/*Eco*RV-cut pCITE-1 carrying IRES (internal ribosome entry site). The resulting plasmid was digested by *Eco*RI to release IRES/p40 fragment which was cloned into *Eco*RI-cut pΔE1sp1A/p40 from pΔE1sp1A/IL-12. *Bam*HI/*Sal*I fragment containing CMV promoter, p35, IRES p40, and poly(A) signal was cloned into *Hind*III-cut adenovirus plasmid pMV60 to form pMV60/IL-12. pMV60 and pJM17 were cotransfected into 293 cells, and plaque was screened to obtain RAdIL-12.

Peptide synthesis

Peptide E1 121–135 was synthesized manually by the solid-phase method of Merrifield (27) using the *N*-fluorenylmethoxycarbonyl alternative (28). The ninhydrin test of Kaiser et al. (29) was used to monitor every step. At the end of the synthesis, peptide was cleaved, deprotected, and washed six times with diethyl ether. The purity of peptide was analyzed by HPLC.

Immunization experiments

Groups of BALB/c mice were immunized i.p. with a combination of different doses of the recombinant adenoviruses RAdCE1, RAd-IL12, and recombinant defective adenovirus expressing *LacZ* gene (RAdLacZ) (see below). Serum samples were extracted at days 3, 6, 11, and 30 after immunization to measure IL-12, IFN-γ, and humoral responses. Thirty days after the immunization, mice were sacrificed, and the CTL response against HCV E1 as well as the intensity and cytokine profile of the Th response against HCV core were analyzed. In some experiments, 25 mg/kg *N*-nitro-L-arginine methyl ester (L-NAME; Sigma Chemical, St. Louis, MO) was administered to mice i.p. at day 0 and the following 6 days after immunization.

In vitro cytokine production in response to HCV core protein

Mice were sacrificed 30 days after immunization, and spleen cells were plated at 0.5×10^6 cells/well in a total volume of 200 μl of culture medium in the presence or absence of 1 μg of core protein. After 24 h, 50 μl of the culture supernatants were removed to measure the presence of IL-2 using a CTL.L bioassay (30). After 48 h of culture, 100 μl of supernatant were removed to measure IFN-γ (ELISA; Genzyme, Cambridge, MA) and IL-4. IL-4 concentrations in the supernatants were measured using a CT4S bioassay (CT4S cells require the presence of IL-4 for their proliferation and were kindly provided by Dr. W. E. Paul and Dr. C. Watson).

Serum levels of cytokines and in vitro production of IL-12 and IFN-γ by resident peritoneal cells

Serum concentration of IL-12 and IFN-γ was measured by commercially available ELISA assays (Genzyme) according to the manufacturer's instructions. In selected experiments, resident peritoneal cells were obtained by lavage of the peritoneal cavity of mice 3 days after i.p. administration of different doses of RAdIL-12. Cells were plated at 1.5×10^5 cells/well in a total volume of 200 μl of culture medium. After 24 h of culture, IL-12 and IFN-γ released to the supernatant were measured by ELISA.

CTL response against E1 121–135

Thirty days after immunization, spleens were removed and homogenized, and cells were cultured in vitro in 24-well plates at 4×10^6 cells/ml in the presence of a final concentration of 5 μg/ml peptide E1 121–135. Culture medium consisted of RPMI 1640 supplemented with 10% FCS, L-glutamine (2 mM), antibiotics, HEPES (5 mM), and 5×10^{-5} M 2-ME. Six days later, cytotoxic activity was measured using a conventional cytotoxicity assay (31). Assays were done in triplicate at different E:T ratios. Spontaneous ^{51}Cr release was in all cases below 25% of total release. P815 mastocytoma cells incubated with or without E1 121–135 peptide were used as targets.

Limiting dilution analysis

Four concentrations of spleen cells (5×10^5, 2.5×10^5, 1.25×10^5, and 0.75×10^5) were placed in 24 replica cultures (for each dilution) in culture medium in the presence of 10 μg/ml peptide E1 121–135, complemented with graded numbers of irradiated syngenic spleen cells (3000 rads) to give a total number of 5×10^5 cells/well (96-well U-bottom plates) in a final volume of 250 μl of medium. After 6 days of culture at 37°C and 5% CO_2, CTL activity of each individual well was measured using the ^{51}Cr release assay by transferring 100 μl of each well in a plate containing radiolabeled P815 cells and peptide E1 121–135, and another 100 μl in a plate with P815 radiolabeled cells without peptide. After 5 h of culture, 50 μl of supernatant were removed from each well, and the percentage of lysis was estimated in a scintillation counter (Topcount, Packard, Meriden, CT). Positive responses for the individual wells were considered when CTL activity in the presence of peptide was 15% higher than in the absence of peptide. Cytotoxic T cell precursor frequencies were calculated on the regression curve by interpolating the number of responder cells required to give 37% negative cultures. Only those experiments were considered for which the data fit in the single-hit model (32) (evaluation according to χ^2).

Measurement of Abs against adenoviral Ags by ELISA Ab subtypes

Anti-adenoviral Abs were titrated by ELISA using microtiter wells previously coated with adenoviral Ags (whole viruses). Plates were incubated with a solution of PBS containing 1% powdered milk and 0.1% Tween 20 (PBSMT) during 2 h at room temperature to block nonspecific Ab binding. After removal of the PBSMT, 50 μl of different serum dilutions were added and incubated at 37°C for 1 h, then washed three times with PBSMT, and incubated at 37°C for 1 h with a PBSMT solution containing 1/1000 goat anti-mouse whole IgG (Amersham International, Little Chalfont, U.K.), 1/200 anti-mouse IgG1 or 1/50 anti-IgG2A biotinylated Abs (Caltag, San Francisco, CA). After three washings with PBSMT, wells were incubated with a 1/500 dilution of horseradish peroxidase-streptavidin (Amersham) at room temperature for 1 h. After three washings with PBS, the color reaction was started by adding 100 μl of a solution prepared by mixing: 10 ml of 0.6% acetic acid (pH 4.7); 5 μl of 33% (w/v) hydrogen peroxidase; and 100 μl of 40 mM water solution of 2,2′-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) (Sigma). After 30 min, the plates were read at 405 nm in a Titertek Multiscan MKII (Flow Laboratories, Puteaux, France).

Measurement of NO in the sera of animals

Nitrite and nitrate were measured using an NOA 280 chemiluminescence detector following the method recommended by Sievers (Sievers Instruments, Boulder, CO). Serum nitrite and nitrate were reduced to NO by incubation with 1 N HCl containing 50 mM VCl_3 at 90°C. The resulting NO was measured by the chemiluminescence derived from its reaction with ozone, according to the following two reactions:

$$NO + O_3 \rightarrow NO_2^* + O_2$$

$$NO_2^* \rightarrow NO_2 + h\nu$$
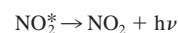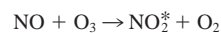
Glutathione levels in spleen

Glutathione and glutathione disulfide content was measured by HPLC according to the method described by Reed et al. (33). Briefly, 200 μl of the cytosolic fraction obtained as indicated above were immediately derivatized using 1-fluoro-2,4-dinitrobenzene. Samples were then chromatographed on a 3-aminopropyl-Spherisorb, 20-cm × 4.6-mm × 5-mm HPLC column, equilibrated in 80% methanol. Elution was conducted with 0.5 M sodium acetate in 64% methanol and followed at 365 nm. Analysis of the chromatogram was performed with Beckman System Gold software.

Immunohistochemistry and measurement of apoptosis

Three groups of four mice were immunized i.p. with 1×10^8 plaque-forming units (pfu) of RAdCE1 in combination with 1×10^8, 1×10^6, or 0 pfu of RAdIL-12. RAdLacZ was administered in such a way that the total dose of adenovirus in each group of mice was 2×10^8 pfu. One mouse per group was sacrificed at days 3, 6, 11, and 16. Spleen specimens from the different groups of animals were cryopreserved in OCT. Measurement of apoptosis was conducted in samples obtained at day 3 using the In situ Cell Death Detection Kit, POD (Boehringer Mannheim, Mannheim, Germany) according to manufacturers instructions. Briefly, tissue sections were fixed with 4% paraformaldehyde, endogenous peroxidase blocked (0.3% H_2O_2 in methanol), and permeabilized by incubation with a solution containing 0.1% Triton X-100. Samples were labeled with fluoresceinated nucleotides by terminal deoxynucleotidyl transferase. Incorporated fluorescein was detected by anti-fluorescein Ab Fab fragments conjugated with peroxidase. Signal conversion was revealed with a Tris buffer containing diaminobenzidine and H_2O_2. Samples were counterstained with methyl green before analysis by light microscope. The number of total and apoptotic nuclei was counted in six randomly selected fields (93292 μm^2 per field) in each tissue section, and the percentage of apoptosis was calculated. A mean of 1530 ± 288 nuclei per tissue section were counted. On day 16 after immunization, spleens from mice immunized with RAdCE1, and different doses of RAdIL-12 were examined by the indirect immunoperoxidase technique using fluoresceinated anti-CD4$^+$ and anti-CD8$^+$ mAbs (Sigma). Incorporated fluorescein was detected by anti-fluorescein Ab Fab fragments conjugated with peroxidase as described above.

Results

Coinjection of RAdCE1 and RAdIL-12: adenoviral gene transfer of IL-12 modulates T cell response against HCV Ags in a dose-dependent manner

Five groups of four BALB/c mice were immunized with 1×10^8 pfu of RAdCE1 together with increasing doses of RAdIL-12 (0, 1×10^5, 1×10^6, 1×10^7, and 1×10^8 pfu). RAdLacZ was injected to complete a total dose of 2×10^8 pfu of recombinant adenoviruses in all groups of animals. Thirty days after immunization, spleen cells from these groups of mice were cultured in the presence or absence of HCV core protein, and the production of IL-2, IFN-γ, and IL-4 was determined.

Fig. 1*A* shows that the production of IL-2 in response to HCV core increased progressively in animals that received 1×10^5–1×10^7 pfu of RAdIL-12 in conjunction with RAdCE1, indicating that within this dose range, codelivery of IL-12-expressing vector and Ag-expressing vector caused a dose-dependent immunostimulatory effect of specific T cell immunity against HCV core. However, we found that when the dose of RAdIL-12 was increased to 1×10^8 the production of IL-2 in response to HCV core decreased abruptly. Similarly, the production of IFN-γ increased when RAdIL-12 was given at doses between 1×10^5 and 1×10^7 pfu but decreased markedly at doses of 1×10^8 pfu (Fig. 1*B*). The concentrations of IL-4 in culture supernatants were undetectable in all groups, indicating that the T helper response against HCV core induced by codelivery of 1×10^8 pfu of RAdCE1 and RAdIL-12 (1×10^5–1×10^7 pfu) was of the Th1 type. Thus, whereas moderate or low doses of RAdIL-12 stimulate Th1-type responses, high doses of the vector depress this type of T cell reactivity.

CTL response against HCV E1 121–135 peptide

In a previous work (20), we showed that mice injected with RAdCE1 developed a CTL response against multiple epitopes from HCV core and against a single epitope (E1 121–135) from the HCV envelope protein E1. In the present study, we analyzed whether different doses of RAdIL-12 (from 1×10^5 to 1×10^8 pfu) could modify the intensity of the CTL response against E1 121–135 induced by a suboptimal dose of RAdCE1 injected simultaneously. As shown in Fig. 1*C*, the magnitude of the CTL response against E1 121–135 increased progressively with doses of RAdIL-12 between 1×10^5 and 1×10^7 pfu. However, when
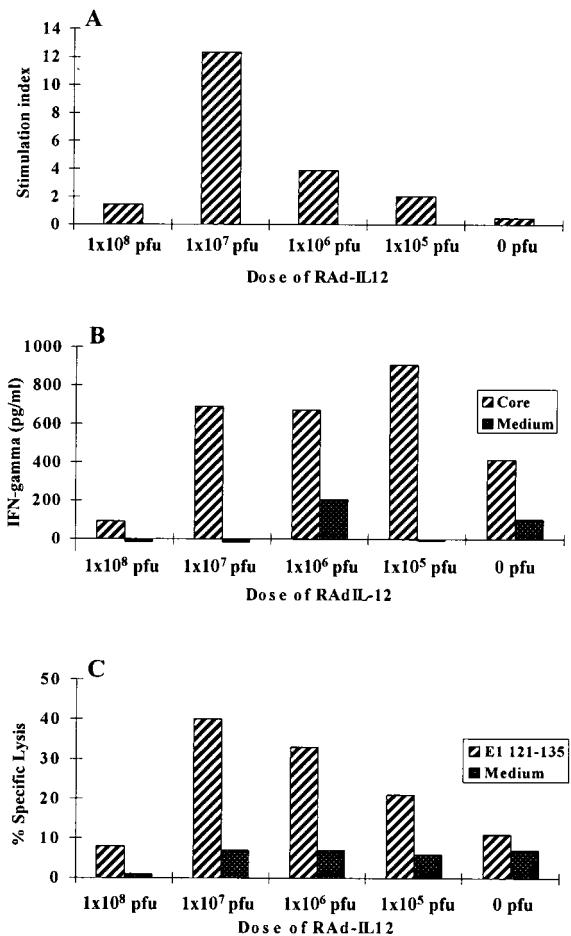

FIGURE 1. Induction of cellular immune responses against HCV Ags after immunization with RAd-IL12. Production of IL-2 (*A*) and IFN-γ (*B*) after restimulation of spleen cells with HCV core protein. *C*, CTL activity against peptide E1 121–135. All mice were immunized with 1×10^8 pfu of RAdCE1 together with different doses (1×10^8, 1×10^7, 1×10^6, 1×10^5, or 0 pfu) of RAdIL-12. Complementary doses of RAdLacZ were administered in such a way that all animals received a total dose of 2×10^8 pfu of recombinant adenoviruses. Supernatants were tested after 24 h (IL-2) and 48 h (IFN-γ) of cell culture in the presence or absence of HCV core protein. Spleen cells were restimulated with peptide E1 121–135 for 5 days, and CTL activity was measured against radiolabeled P815 cells incubated with or without peptide E1 121–135. An E:T ratio of 20:1 was used.

RAdIL-12 was used at the dose of 1×10^8 pfu a marked fall in CTL activity occurred. Thus, in agreement with results of T helper responses, we found that doses of RAdIL-12 between 1×10^5 and 1×10^7 pfu had an important adjuvant effect on induction of specific CTL activity whereas high doses of the IL-12 expressing vector markedly inhibited CTL generation.

Induction of Abs against adenoviral Ags: Ab subtypes

As observed by others (34, 35), we could not detect Abs against HCV core in any of the experimental groups of animals, possibly because this protein is not readily secreted out of the cell. Thus, to evaluate the effects of different doses of RAdIL-12 on humoral immunity, we analyzed the changes in Ab production against the adenoviral vector. Anti-adenovirus Ab titers were determined by ELISA in serum samples obtained at days 6, 11, and 30 after immunization with RAdCE1 and different doses of RAdIL-12 as previously described. Total IgG, IgG1, and IgG2A Ab subtypes
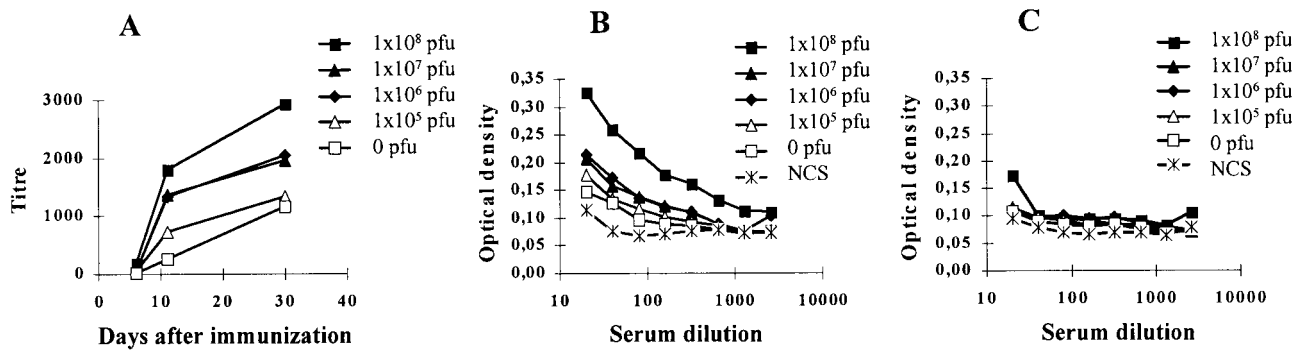
FIGURE 2. Humoral responses against adenoviral particles. Anti-adenoviral total IgG Abs (*A*), IgG2A (*B*), and IgG1 (*C*) were measured by ELISA using plates coated with whole adenovirus particles. Titration of anti-adenoviral total IgG was carried in all groups of animals at days 6, 11, and 30 after immunization. As in Fig. 1, all mice were immunized with 1×10^8 pfu of RAdCE1 together with different doses (1×10^8, 1×10^7, 1×10^6, 1×10^5, or 0 pfu) of RAdIL-12. Complementary doses of RAdLacZ were administered in such a way that all animals received a total dose of 2×10^8 pfu of recombinant adenoviruses. Measurement of IgG2A and IgG1 was conducted at day 30 after immunization. Results are the mean of three animals per group. NCS, negative control serum.

were measured using specific secondary Abs (IgG1 and IgG2A subtypes were determined only on day 30).

Fig. 2*A* shows that total IgG Ab titers increased progressively with increasing doses of RAdIL-12, with the highest values being observed with the greatest dose of this vector (1×10^8 pfu). IgG2A (Th1 dependent) Abs followed the same pattern (Fig. 2*B*) whereas no IgG1 Abs (Th2 dependent) were detected (Fig. 2*C*). It can be concluded that immunization with RAdIL-12 potentiates humoral response against adenoviral Ags and that this response is compatible with the induction of a Th1 profile. Interestingly, whereas high doses of RAdIL-12 resulted in abrogation of T cell responses, humoral immunity was not suppressed but rather considerably stimulated by high doses of the vector.

Concentrations of IL-12 and IFN-γ in serum and in vitro production of cytokines by resident peritoneal cells

Serum levels of IFN-γ and IL-12 were measured on days 3, 6, 11, and 30 after coimmunization with RAdCE1 and different doses of RAdIL-12. As represented in Fig. 3*A*, mice that received 1×10^6 and 1×10^7 pfu of RAdIL-12 showed a small peak of IFN-γ in serum (below 200 pg/ml) between day 3 and day 11 with maximal values on day 6. In these animals, the levels of circulating IL-12 were undetectable (Fig. 3*B*) in all determinations. In contrast, a peak of near 300 pg/ml of IL-12 was found on day 3 in mice injected with 1×10^8 pfu of RAdIL-12. These mice showed very high concentrations of IFN-γ in serum on days 3 and 6 with maximal values around 1200 pg/ml on day 6 (Fig. 3*A*). These data indicate that increased systemic levels of IL-12 and marked elevation of IFN-γ in serum occur only with high doses of RAdIL-12, the ones that cause suppression of specific T cell responses. Low or intermediate doses of the vector cause enhancement of T cell immunity without increasing circulating IL-12 and with only mild elevations of IFN-γ in serum. From the results obtained in animals injected with 1×10^8 pfu of RAdIL-12, it seems that increased production of IL-12 occurs first (peak value on day 3) to be followed by enhanced production of IFN-γ (peak value on day 6).

We also analyzed the effect of RAdIL-12 on cytokine production by resident peritoneal cells (1.5×10^5 cells maintained in culture for 24 h) by determining the spontaneous release of IL-12 and IFN-γ by these cells 3 days after i.p. injection of different doses (1×10^8, 1×10^7, and 0 pfu) of the vector. IL-12 was detectable (54 pg/ml) only in peritoneal cell supernatants from animals immunized with 1×10^8 pfu of RAdIL-12. IFN-γ concentrations in culture supernatant were 770, 481, and 358 pg/ml

(mean of three mice per group) in animals that received 1×10^8, 1×10^7, and 0 pfu of RAdIL-12, respectively, indicating a dose-dependent stimulatory effect of RAdIL-12 on IFN-γ production.

Production of NO after immunization with RAdCE1 and different doses of RAdIL-12

We determined the levels of nitrites and nitrates in serum as an estimation of the amount of NO generated. As represented in Fig. 4, immunization with RAdCE1 in conjunction with high doses of RAdIL-12 (1×10^8 pfu) resulted in a marked increase in the production of NO. In this group, the highest values (211 μM) were
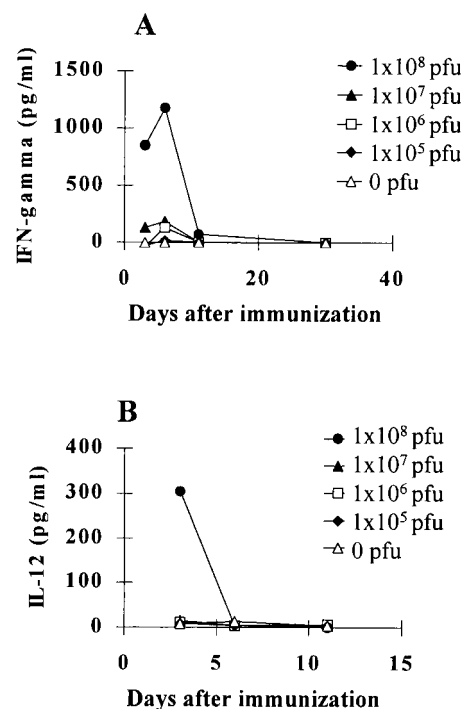

FIGURE 3. Levels of IFN-γ (*A*) and IL-12 (*B*) in the sera at days 6, 11, and 30 following immunization of animals with RAdCE1 and various doses of RAdIL-12 or RAdLacZ alone. All mice were immunized with 1×10^8 pfu of RAdCE1 together with different doses (1×10^8, 1×10^7, 1×10^6, 1×10^5, or 0 pfu) of RAdIL-12. Complementary doses of RAdLacZ were administered in such a way that all animals received a total dose of 2×10^8 pfu of recombinant adenoviruses.
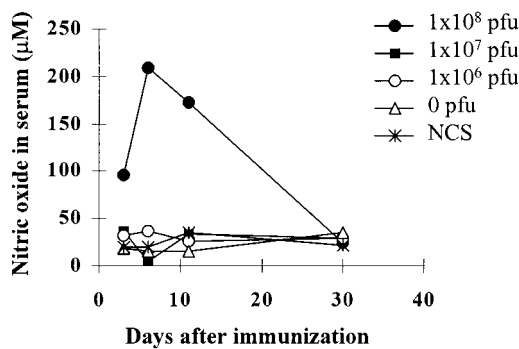
FIGURE 4. Levels of NO (μM) in the sera at days 3, 6, 11, and 30 after immunization with RAdCE1 and various doses of RAdIL-12 or RAdLacZ alone. NCS, negative control serum. All mice were immunized with 1×10^8 pfu of RAdCE1 together with different doses (1×10^8, 1×10^7, 1×10^6, 1×10^5, or 0 pfu) of RAdIL-12. Complementary doses of RAdLacZ were administered in such a way that all animals received a total dose of 2×10^8 pfu of recombinant adenoviruses.

observed at day 6 and returned to concentrations found in untreated mice by day 30 after immunization. Administration of RAdCE1 together with lower doses of RAdIL-12 (1×10^7 and 1×10^6 pfu) did not induce any detectable rise of NO in serum. In these groups, the levels were similar to those observed with the control adenovirus RAdLacZ (0 pfu of RAdIL-12).

Cell apoptosis in the spleen

Both abrogation of T cell responses and increased NO production were found in mice treated with 1×10^8 pfu of RAdIL-12. Because NO has been shown to induce apoptosis of T cells (36), we decided to analyze the presence of apoptotic cells in the spleen of animals immunized with different doses of RAdIL-12. Mice received i.p. 1×10^8 pfu, 1×10^6, or 0 pfu of RAdIL-12 (RAdLacZ was used as control adenovirus to complete a total dose of 2×10^8 pfu of adenovirus in all animals). The study was performed on day 3 after vector administration. The quantitation of apoptosis was performed by examination of tissue sections stained using the TUNEL technique. As shown in Fig. 5, the percentage of apoptotic cells per field in the spleen is higher in animals treated with 1×10^8 pfu of RAdIL-12 than in the other two groups.

In addition, immunohistochemistry of CD4$^+$ and CD8$^+$ cells performed in tissue sections of the spleen obtained on day 16 after immunization revealed the disappearance of CD4$^+$ and CD8$^+$ T
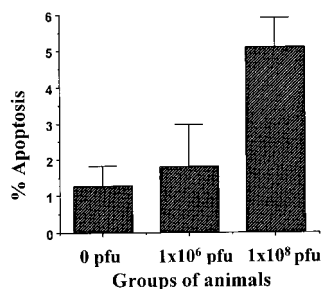

FIGURE 5. Percent of apoptosis in spleen cells after immunization with different combinations of recombinant adenoviruses. Mice were immunized i.p. with 1×10^8, 1×10^6 or 0 pfu of RAd IL-12. RAdLacZ was administered in such a way that the total dose of adenovirus in each group was 2×10^8 pfu. Apoptosis was measured at day 3 after immunization. Percentage of apoptosis was calculated from the ratio between the total nuclei and the apoptotic nuclei in six randomly selected fields from each tissue section.

cells in mice immunized with RAdCE1 and 1×10^8 pfu of RAdIL-12 but not in those who received a lower dose of the IL-12-expressing vector (Fig. 6).

Effect of L-NAME administration on the induction of T cell responses

In preliminary experiments, we found that administration of L-NAME (an inhibitor of inducible NO synthase) caused death within the first 3 days after immunization of all animals that received RAdCE1 and 1×10^8 pfu of RAdIL-12. Thus, we investigated the effect of L-NAME in animals immunized with RAdCE1 (1×10^8 pfu) and lower doses of RAdIL-12 (5×10^7 and 1×10^7 pfu). RAdLacZ (4×10^7 pfu) was used to complete a total dose of adenovirus of 1.5×10^8 pfu in the group that received the lower dose of RAdIL-12. Three animals per group were treated by i.p. injection of 100 μg/day/animal of L-NAME in saline (Table I). Determinations of NO and IFN-γ were done in serum samples on day 6 after immunization and spleen cells were obtained on day 30 to analyze cellular immune responses.

Mice that received high doses of RAdIL-12 (5×10^7 pfu) exhibited very high serum values of IFN-γ and increased NO levels but survived normally. Similarly to what we found in animals treated with 1×10^8 pfu of RAdIL-12, administration of L-NAME caused 100% mortality in the group of mice that received 5×10^7 pfu of RAdIL-12. In contrast, less marked elevations of IFN-γ and NO were found in animals immunized with a lower dose of RAdIL-12 (1×10^7 pfu). These mice did not experience apparent toxicity after L-NAME administration. However, in this group L-NAME produced a reduction of serum IFN-γ to undetectable levels and induced a marked inhibition of both CTL activity against HCV E1 121–135 peptide and proliferative response to HCV core (Table I). In addition, as shown in Fig. 7, L-NAME caused a substantial decrease in the production of Abs to adenoviral Ags, both total IgG and IgG2A Abs. Finally, the immunization protocol with RAdCE1 plus 1×10^7 RAdIL-12 caused oxidative stress as manifested by low levels of glutathione and total glutathione in the spleen (Table I). This effect was suppressed, together with specific immune response to Ags, by treatment with L-NAME.

Discussion

The development of a specific Th1 type of response is critical in the elimination of intracellular pathogens such as viruses, protozoa, and different types of bacteria (1). IL-12 is produced by APCs (37) and plays an important role in the induction of Th1 responses through its ability to prime naive Th0 cells for high IFN-γ production (3, 38). Thus, IL-12 has been considered to have potential as an adjuvant in prophylactic and therapeutic vaccines (2).

IL-12 can be applied as a recombinant protein (rIL-12) or by means of gene transfer methodologies. Administration of rIL-12 may produce systemic toxicity, and in fact this cytokine is known to have a narrow therapeutic window (16). Gene transfer methodologies might favor low level sustained production of the cytokine at the right site allowing stimulation of T cell responses in the absence of toxicity (18, 19). Since the immunomodulatory effects of rIL-12 have been shown to be dose-dependent (14), this dose dependency might also occur when using gene transfer techniques. Thus, we have used two adenoviral vectors one expressing HCV core and E1 (RAdCE1) and the other expressing IL-12 (RAdIL-12) to study the effects of different doses of RAdIL-12 on the induced immune response against HCV Ags.

Our results indicate that low and intermediate doses of RAdIL-12 activate a Th1 type of response against HCV proteins while high doses resulted in a marked inhibition of the T cell
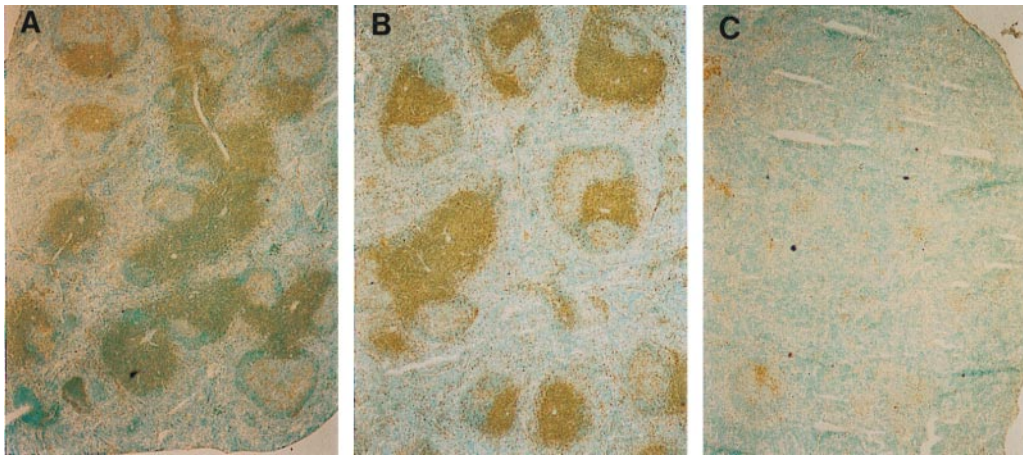
FIGURE 6. Staining of CD4$^+$ and CD8$^+$ cells in spleen tissue sections from mice immunized with 1×10^8 pfu of RAdCE1 in conjunction with 0 pfu (*A*), 1×10^6 pfu (*B*), or 1×10^8 pfu (*C*) of RAdIL-12. RAdLacZ was administered in such a way that the total dose of adenovirus in each group was 2×10^8 pfu. Magnification was $\times 20$ in all cases.

response to HCV Ags with abolition of CTL activity against E1, and profound decrease of IL-2 and IFN-γ production in the presence of core Ag. The results discussed above are in agreement with previous data in the literature showing that low doses of rIL-12 activate specific Th1 response in mice infected with lymphocytic choriomeningitis virus while high doses cause suppression of specific T cell responses and increased viral load (14).

Of importance, in our model, low and intermediate doses of RAdIL-12 induced an important enhancement of Th1 responses without any changes in circulating IL-12 levels, suggesting that this method can produce immunostimulatory effects without the risk of side effects derived from increased cytokinemia as observed in treatments with rIL-12 (16). In our study T cell immunosuppression with high doses of RAdIL-12 was associated with considerable elevation in circulating IL-12 on day 3 after immunization and with very high levels of IFN-γ in serum with the greatest values being found on day 6 (Fig. 3). The kinetics of these cytokines suggest that IL-12 is expressed early by RAdIL-12-transduced cells and increased production of IL-12 stimulates IFN-γ generation. The stimulatory effect of RAdIL-12 on IFN-γ generation was also evidenced in cell cultures from resident peritoneal cells. i.p. administration of RAdIL-12 induced detectable production of IL-12 by resident peritoneal cells only at the highest dose tested (1×10^8) but it had a clear stimulatory dose-dependent

effect on the spontaneous production of IFN-γ by these cells, indicating that IL-12 below the detection limit can efficiently enhance IFN-γ production. Since IFN-γ was shown to enhance NO production (15, 39) and NO has recently emerged as a potent regulator of lymphocyte function (21–26) we have analyzed the role of this substance in the immunoregulatory effects induced by RAdIL-12.

We found that production of NO did not change in animals treated with low or intermediate doses of RAdIL-12 but increased markedly in mice which received high doses of the vector. These observations are in agreement with reports indicating that rIL-12 stimulates in a dose-dependent manner the in vitro production of IFN-γ and NO by splenocytes (15). The same authors proved that anti-IFN-γ antibodies were effective in preventing the rise of NO induced by IL-12. In our system the changes of NO and IFN-γ in serum occur in parallel suggesting a role of IFN-γ in NO generation.

NO has been shown to cause immunosuppression and to reduce the production of Th1 cytokines when generated in big amounts (22, 40). In fact, NO has been implicated in the pathogenesis of immunosuppression caused by *S. typhimurium* (15) and malarial (41) infections. Different studies have indicated that Th1 but not Th2 cells are sensitive to the inhibitory effects of NO (22, 42, 43). In addition, thymocytes (36) can undergo NO-induced apoptosis

Table I. *Effects of L-NAME administration on survival, immune response to HCV Ags, concentrations of NO and IFN-γ in serum and glutathione in spleen cells[a]*

| Dose of RAdIL-12 | Treatment | % of Survival | NO in Serum (μM)[b] | IFN-γ in Serum (pg/ml)[b] | Glutathione[c] | | | Frequency of CTL Precursors[d] | IL-2 Production[e] |
					GSH	GSSG	Total		
5×10^7	None	100 (3/3)	65.5 ± 10.2	993.6	ND	ND	ND	ND	ND
	L-NAME	0 (0/3)							
1×10^7	None	100 (3/3)	26.5 ± 3.5	687.3	15.47	1.58	17.05	1/114548	15503 (3073)
	L-NAME	100 (3/3)	13.6 ± 2.5	<38	24.04	2.64	26.73	<1/10^6	3994 (1539)
None	None	100 (3/3)	13.2 ± 3.2	<38	21.10	1.62	22.62	ND	ND

[a] Two groups of six animals were immunized with 1×10^8 pfu of RAdCE1 in conjunction with either 5×10^7 pfu of RAdIL-12 or 1×10^7 pfu of RAdIL-12 + 4×10^7 pfu of RAdLacZ. Three animals per group were treated by i.p. administration of 100 μg/day/animal of L-NAME in saline. A nonimmunized control group was also included in these experiments.

[b] NO and IFN-γ was measured in the sera extracted from mice 6 days after immunization.

[c] Glutathione (GSH) concentrations were measured in spleens of animals extracted 6 days after immunization. GSSG, glutathione disulfide.

[d] Frequency of CTL precursors against target cells incubated with peptide E1 121–135 was measured 30 days after immunization.

[e] IL-2 production of spleen cells incubated with HCV core protein (thymidine incorporation of CTLL cells in presence of supernatants of spleen cells incubated with or without (in parentheses) HCV core protein) was measured 30 days after immunization.
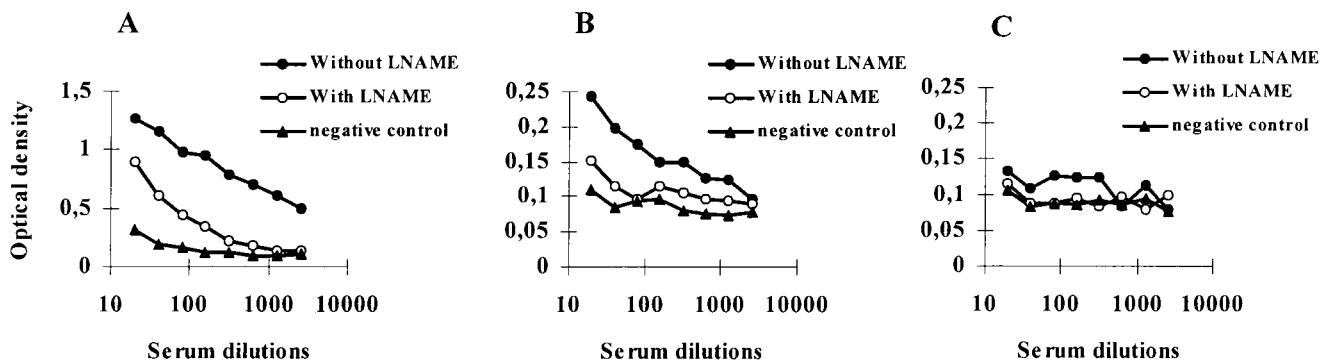
FIGURE 7. Effect of L-NAME on the induction of Abs against adenoviral Ags. Untreated mice (□) or treated with L-NAME (■) were immunized with 1×10^7 pfu of RAdIL-12. Titers of Abs against adenoviral Ags were measured at day 30 after immunization. *A*, total IgG; *B*, IgG2A; *C*, IgG1 Abs.

while NO can play a protective role against apoptosis of B lymphocytes (44, 45). These data offer an explanation for findings in the present study which show that animals treated with high doses of RAdIL-12 and generating elevated levels of NO develop suppression of specific T cell immunity (Figs. 1A, 1B, 1C, and 6) while humoral response to adenoviral Ags was enhanced in a dose-related manner with the highest Ab production corresponding to the greatest dose of RAdIL-12 (Fig. 2). Since the induction of IgG2A Abs is Th1 dependent and this type of Abs was also stimulated by high doses of RAdIL-12 which appear to be inhibitory for Th1, we could hypothesize that priming of B cells could occur during the first days after immunization, before NO generation reached levels inhibitory for Th1 function.

L-NAME, an inhibitor of inducible NO synthase, did not produce any apparent toxicity in mice which received RAdIL-12 at doses of 1×10^7 pfu but caused mortality in all animals treated with 1×10^8 or with 5×10^7 pfu. In the absence of L-NAME these mice had very high IFN-γ and increased NO values but survived normally, indicating the already described protective role of NO in the hypercytokinemic syndrome (46). A relevant finding in this study relates to the observation that NO, when produced in relatively low amounts, appears to play a critical role in T and B cell activation in vivo. As indicated in Results, animals immunized with immunostimulatory doses of RAdIL-12 (1×10^7 pfu) in combination with RAdCE1 and treated with L-NAME failed to develop T cell proliferation in response to HCV core, exhibited a profound fall in IFN-γ production and the frequency of CTL precursors against E1 121–135 decreased to less than $1/10^6$ (Table I). Moreover, immunization with the combination of adenoviral vectors in the presence of L-NAME resulted in a substantial decrease in the production of anti-adenoviral Abs. These results strongly implicate NO as a mediator in T cell activation and in the development of both cellular and humoral immunity after coimmunization with IL-12. Although NO has been reported to stimulate cytokine production in vitro (47, 48) there are no previous data in the literature showing the inhibitory effect of NO synthase inhibitors on IL-12-stimulated T and B cell responses in vivo.

In our study we observed a reduction in glutathione concentration in the spleen in mice treated with RAdCE1 and 1×10^7 pfu of RAdIL-12, whereas glutathione values similar to controls were detected in L-NAME-treated mice subjected to the same immunization procedure (Table I). These findings are consistent with the presence of NO-mediated oxidative stress in IL-12-treated mice which might have a role in immune activation. In the same direction, a recent report has shown that glutathione levels in APCs play a role in Th1-associated cytokine production (49). Recent data indicate that oxidative stress participates in activation of both T

and B cells by mechanisms involving induction of thioredoxin and NF-κB translocation (50).

In conclusion, coadministration of two adenoviral vectors, one expressing HCV Ags and the other IL-12, resulted in enhancement of specific T and B cell immune responses to HCV Ags with a Th1 profile when the IL-12-expressing vector was used at low or intermediate doses. However, marked suppression of specific T cell immunity occurred with high doses of IL-12-producing vector. NO appears to play an important role in both immunostimulatory and immunosuppressive effects of IL-12. Low doses of recombinant adenoviruses expressing IL-12 induce potent adjuvant effects without increasing serum levels of IL-12 or IFN-γ and thus likely without the side effects of hypercytokinemia which may follow administration of rIL-12.

Acknowledgments

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