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Vaccine 23 (2005) 3493–3499
Vaccine
www.elsevier.com/locate/vaccine

Enhancement of CD4 and CD8 immunity by anti-CD137 (4-1BB) monoclonal antibodies during hepatitis C vaccination with recombinant adenovirus

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Received 9 September 2004; received in revised form 19 January 2005; accepted 4 February 2005
Available online 8 March 2005

Abstract

The induction of protective or therapeutic cellular immunity against hepatitis C virus (HCV) is a difficult goal. In a previous work we showed that immunization with a recombinant adenovirus encoding HCV-NS3 (RAdNS3) could partially protect mice from challenge with a vaccinia virus encoding HCV antigens. We sought to investigate whether systemic administration of an immunostimulatory monoclonal antibody directed against the lymphocyte surface molecule CD137 could enhance the immunity elicited by RAdNS3. It was found that treatment with anti-CD137 mAb after the administration of a suboptimal dose of RAdNS3 enhanced cytotoxic and T helper cell responses against HCV NS3. Importantly, the ability of RAdNS3 to induce protective immunity against challenge with a recombinant vaccinia virus expressing HCV proteins was markedly augmented. Thus, combination of immunostimulatory anti-CD137 mAb with recombinant adenoviruses expressing HCV proteins might be useful in strategies of immunization against HCV.

Keywords: 4-1BB; Vaccination; Hepatitis C virus

1. Introduction

Infection by HCV is the major cause of non-A, non-B hepatitis, affecting an estimated 170 million people worldwide [1]. It is characterised by a high tendency to chronicity, which often progresses to cirrhosis and liver cancer [2]. Viral clearance after acute hepatitis or after IFN-α therapy is usually associated with strong CD4 and CD8 T cell responses [3–12]. In particular, cellular T helper immune response against non-structural NS3 HCV protein has been associated with viral clearance after acute infection, whereas absence of this T-cell response leads to viral persistence and chronic hepatitis [9,13]. Several studies have also detected cytotoxic T cell responses against NS3 protein in HCV patients [14–16]. Thus, NS3 might be a good target for the development of anti-HCV prophylactic or therapeutic vaccination.

Recombinant viruses constitute an attractive strategy to induce strong cellular immune responses. In a previous work we showed that the replication-deficient adenovirus RAdNS3 expressing HCV NS3 protein was able to induce both $CD4^+$ and $CD8^+$ cellular immunity [17]. When mice were immunized with 1×10^9 pfu of RAdNS3, 60% of the animals were protected against challenge with a recombinant vaccinia virus expressing HCV proteins (vHCV1-3011). However, when the dose of RAdNS3 was reduced to 5×10^8 pfu, only a marginal

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0264-410X/$ – see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2005.02.003

induction of anti-NS3 T cell responses were detected and no protection against vHCV1-3011 challenge was elicited. It has been recently described that HCV proteins might impair dendritic cell differentiation and mediate an immunoinhibitory effect on the induction of anti-HCV immune responses [18–20]. Thus, strategies aimed at augmenting the immunogenicity of the recombinant adenovirus expressing NS3 might have important implications for a potential anti-HCV vaccine.

Dendritic cells (DC) are a subset of antigen presenting cells with potent immunostimulatory properties for the induction of T cell responses [21]. These cells capture and process antigens, migrate to lymphoid tissues, express costimulatory molecules and produce cytokines and chemokines that attract and activate T cells. Interaction of these costimulatory molecules with their ligands constitute the needed "second signal" for the full activation of T cells. Thus, combination of an antigen with costimulatory signals on the immunization schedule may have a beneficial effect to enhance cellular immune responses against a defined antigen [22,23]. A number of costimulatory signals provided by members of the TNF receptor superfamily (TNFR) have been shown to augment T cell responses. Among these ligand-receptor pairs at the surface of DC and T cells that may amplify T cell responses, interaction of CD137 with its ligand CD137L, has been shown to activate strongly T cell immunity (reviewed in [24]).

CD137 is expressed on activated, but not resting T cells [25]. Also, CD137 expression has been detected on B cells, macrophages, dendritic cells, activated NK cells, or eosinophils although the physiological significance of the expression on these cell types remains to be elucidated (reviewed in [26]). A single ligand for CD137 has been identified whose expression has been found on activated dendritic cells, B cells and macrophages [27–30]. There is ample evidence that CD137 driven signals are potent for $CD8^+$ T cell stimulation [23,31–40] and if provided together with those signals from CD28 and TCR, the T lymphocytes reach optimal levels of activation [23,34,38,39]. Thus, treatments with agonistic anti-CD137 monoclonal antibodies have shown to induce potent CTL-mediated antitumor effects [31,41] synergizing with tumor antigen immunization [41,42].

In the present study, we have studied whether the administration of anti-CD137 after immunization with a suboptimal dose of RAdNS3 improves the anti-HCV immune response induced in mice. As a surrogate for HCV infection (which does not infect mice), mice immunized with a non-protective dose of RAdNS3 followed by anti-CD137 antibody administration were challenged with a recombinant vaccinia virus expressing HCV polyprotein (vHCV1-3011). As discussed below, we show that co-administration of anti-CD137 antibodies strongly potentiates both $CD4^+$ and $CD8^+$ T cell responses against HCV NS3 protein and achieves full protection of mice from infection with vHCV1-3011.

2. Materials and methods

2.1. Mice

Female BALB/c mice, 4–6 weeks old were purchased from Harlan (Barcelona, Spain), hosted in appropriated animal care facilities during the experimental period and handled following the Institutional guidelines required for experimentation with animals.

2.2. Cells and viruses

Two hundred and ninty-three cells (ECACC no.: 85120602), derived from human embryonic kidney and stably transfected with the E1 region of the adenovirus type 5 (Ad5), were used for generation and propagation of the recombinant adenoviruses. Recombinant adenovirus expressing hepatitis NS3 (HCV) protein (RAdNS3) under the control of the cytomegalovirus major immediate early promoter was generated as described [17]. The recombinant adenovirus was propagated on 293 cells and purified in a CsCl isopicnic banding step. Viral band was dialyzed against 0.01 M Tris, pH 8.0. At this stage, the virus was kept in aliquots at $-80\,^{\circ}C$. P815 cells (ATCC TIB64) were used as target cells for cytotoxicity assays. BSC-1 cells (kindly provided by Dr. J.A. Berzofsky, NIH Bethesda, MD) were used for titration of vaccinia virus in ovaries. These cell lines were cultured in RPMI-1640 medium plus 10% foetal calf serum, antibiotics and 5×10^{-5} M 2-mercaptoethanol at $37\,^{\circ}C$ and 5% CO_2. Vaccinia virus vHCV1-3011 expressing HCV polyprotein was kindly provided by Dr. CM Rice (Washington University School of Medicine, St. Louis, MO).

2.3. NS3 recombinant protein and peptide synthesis

The HCV NS3 helicase domain from HCV H (amino acids 1192–1458) was expressed in *Escherichia coli* and purified as previously described [17]. Peptide p1215, which encompasses an immunodominant cytotoxic T cell epitope within NS3 [17], was synthesized by the solid phase method using a multiple synthesizer as previously described [43], analyzed by HPLC and used without further purification.

2.4. Production and purification of anti-CD137 mAb

A rat IgG2a against murine CD137 was produced as previously described [41]. The protein in the culture supernatants was purified using a HiTrap protein G-sepharose column (Amersham Pharmacia Biotech, Piscataway, NJ) and dialyzed in LPS-free PBS. Endotoxin levels were tested using a Quantitative Chromogenic Limulus Amebocyte Lysate assay (Bio-Whittaker) and were <0.5 EU/μg protein.

2.5. Measurement of cytotoxic T cell activity after immunization with RAdNS3

Mice were immunized i.p. with 5×10^8 pfu of RAdNS3 virus in 500 μl of PBS. Four days after immunization they received an i.p. administration of 100 μg of anti-CD137 monoclonal antibody, 100 μg of rat IgG or saline (three mice per group). Twelve to 15 days after immunization, spleens were removed, homogenized and 8×10^5 cells cultured in 96-well plates in RPMI-1640 medium supplemented with 10% fetal calf serum, antibiotics and 5×10^{-5}M of 2-mercaptoethanol, in the presence or absence of peptide p1215 (10 μg/ml). Five days later, cytotoxic activity was measured as previously described [44] using a conventional cytotoxicity assay using radio-labeled P815 cells pulsed with p1215 as target cells.

2.6. Cytokine measurement

Spleen cells from immunized animals were plated on 96-well U-bottomed plates at 8×10^5 cells/well with culture medium in the presence or absence of NS3 protein (1 μg/ml), or peptide p1215 (25 μg/ml) to a final volume of 0.25 ml. In some experiments, 100 μg/ml of anti-CD4 or anti-CD8 antibodies (obtained from rat anti-mouse hybridomas GK 1.5 and H35 17.2, respectively) were added to the cultures. Supernatants (50 μl) were removed 48 h later for the measurement of IFN-γ. IFN-γ production was measured by ELISA (Pharmingen, San Diego, CA) according to manufacturer's instructions.

2.7. In vivo protection against infection with a recombinant vaccinia virus expressing HCV proteins

Immunized mice were challenged i.p. with 5×10^6 pfu of the recombinant vaccinia vHCV1-3011 expressing HCV polyprotein (10 mice per group). Three days after vaccinia challenge, mice were sacrificed and ovaries removed, weighted, homogenized, sonicated and assayed for vHCV1-3011 titre by plating serial 10-fold dilutions of samples on a plate of BSC-1 indicator cells. After 2 days of culture, cells were stained with crystal violet to detect pfu at each serial dilution.

2.8. Statistics

Statistical analysis was carried out to evaluate the protective effect of adenovirus immunization. Data were analysed by the Kruskal–Wallis test, followed by Mann–Whitney's *U*-test as a multiple comparison test with Bonferroni adjustment. All statistical analyses were performed with SPSS v6.0 for Windows package. All *p*-values were two-tailed.

3. Results

3.1. Anti-CD137 monoclonal antibody administration after immunization with a recombinant adenovirus expressing HCV-NS3 protein enhances $CD4^+$ T cell response against NS3

In a previous study, we showed that i.p. immunization with 10^9 pfu of a recombinant adenovirus expressing HCV-NS3 protein (RAdNS3) was able to induce both T helper and T cytotoxic anti-NS3 responses [17]. The elicited immune response was able to protect around 60% of mice against challenge with the recombinant vaccinia vHCV1-3011 expressing HCV proteins. However, when the dose of adenovirus used for immunization is reduced to 5×10^8 pfu, neither T helper nor T cytotoxic T cell response against NS3 are detected and no protection against vHCV1-3011 was observed. Induction, expansion and maintenance of T cell responses are achieved through intricate and precise interactions between $CD4^+$ T cells, $CD8^+$ T cells and dendritic cells. Among the different ligand-receptor pairs implicated, interaction of CD137L expressed on dendritic cells and CD137 on T cells has been shown to be very important for maintenance and full expansion of activated T cells. For these reasons we tested the capacity of an agonistic anti-CD137 specific monoclonal antibody to enhance the immune response against an antigen injected at a suboptimal dose. Thus, groups of mice were immunized i.p. first with 5×10^8 pfu of RAdNS3 followed by the i.p. administration of 100 μg of anti-CD137. As controls, two groups of RAdNS3 immunized mice were injected with saline or with 100 μg of rat IgG. Also, we included other control group of mice treated only with anti CD137 antibodies. Mice were sacrificed 12–15 days after adenovirus immunization and spleen cells were cultured in the presence or absence of NS3 protein. IFN-γ present in the culture supernatants was measured by ELISA. As it is shown in Fig. 1A, immunization with 5×10^8 pfu of RAdNS3 did not induce T cells able to produce IFN-γ in response to NS3 recombinant protein. However, if RAdNS3 immunization is followed by anti-CD137 administration, spleen cells produce high amounts of IFN-γ in response to NS3. Administration of anti-CD137 in the absence of a previous immunization with RAdNS3, or the administration of rat IgG after RAdNS3 immunization did not have any effect on IFN-γ production. The IFN-γ production was mediated by $CD4^+$ T cells since addition of anti-CD4 to the cultures, but not anti-CD8 or control antibodies, inhibited the production of this cytokine (Fig. 1B).

3.2. Anti-CD137 antibody administration after immunization with RAdNS3 enhances CTL responses against NS3

To study the CTL response induced by RAdNS3 immunization, we measured the CTL activity against peptide p1215, which, as we have previously shown [17], encompasses an immunodominant cytotoxic T cell epitope within

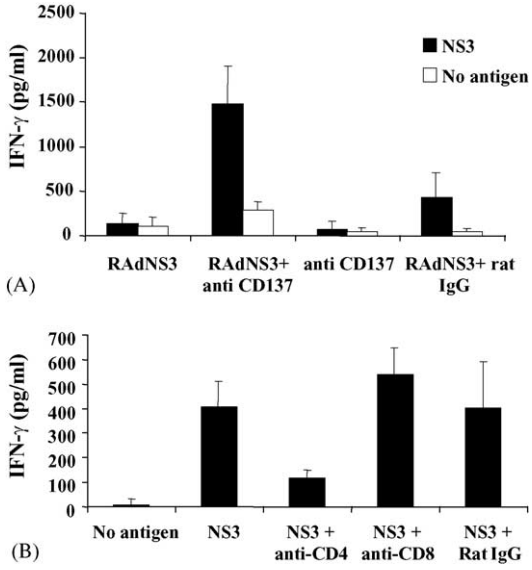

Fig. 1. Administration of anti-CD137 mAb after immunization with RAdNS3 enhances $CD4^+$ T cell response against NS3 protein. (A) Spleen cells from mice immunized with RAdNS3, RAdNS3 plus anti-CD137, anti-CD137 alone or with RAdNS3 plus rat IgG (three mice per group), were cultured in vitro in the presence or absence of recombinant NS3 protein. Culture supernatants were harvested 48 h later and the presence of IFN-γ in these supernatants was measured by ELISA. (B) Spleen cells from mice immunized with RAdNS3 plus anti CD137 were cultured in the absence or presence of NS3. Anti-CD4, anti-CD8 or rat IgG were added to the cultures and IFN-γ secreted to the supernantant was measured by ELISA. Bars represent the mean average of triplicate samples + S.E.M.

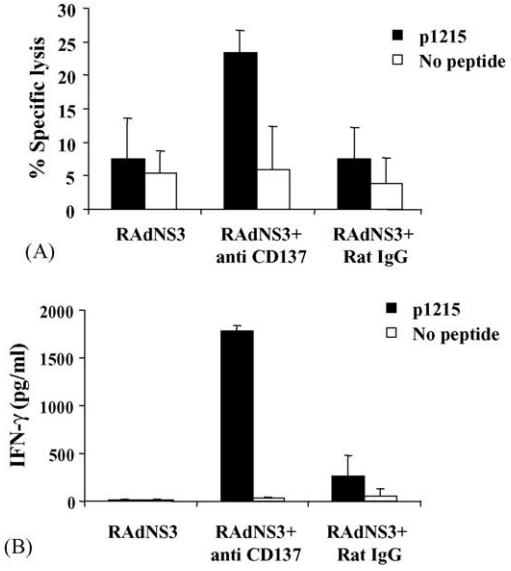

Fig. 2. Administration of anti-CD137 mAb after immunization with RAdNS3 enhances cytotoxic T cell response against peptide p1215 from NS3. Spleen cells from mice immunized with RAdNS3, RAdNS3 plus anti-CD137, or with RAdNS3 plus rat IgG (three mice per group) were cultured in vitro in the presence or absence of 10 μg/ml of peptide p1215 from NS3. (A) After 5 days of culture, CTL activity against P815 target cell pulsed with p1215 peptide was measured using the ^{51}Cr release assay. An effector:target ratio of 25 was used. (B) IFN-γ production after 2 days of culture in the presence or absence of peptide p1215 was measured by ELISA. Bars represent the mean average of triplicate samples + S.E.M.

NS3. Thus, spleen cells from the groups of mice described above were cultured in the presence or absence of peptide p1215. As in the case of the T helper response, we were unable to detect cytolytic activity against P815 target cells pulsed with peptide p1215 in mice who had received 5×10^8 pfu of RAdNS3. However, if RAdNS3 immunization is followed by anti-CD137 administration, a significant increase on CTL activity was observed (Fig. 2A). This enhancement was also observed when we measured the IFN-γ production of spleen cells in the presence of peptide p1215 (Fig. 2B).

3.3. Anti-CD137 antibody administration after immunization with RAdNS3 confers protection against infection with vHCV1-3011 expressing HCV polyprotein

To analyze in vivo the efficacy of immunization with a suboptimal dose of RAdNS3 followed by anti-CD137 administration we measured the protection of mice against infection with vHCV1-3011 expressing HCV polyprotein. Thus, 10 days after adenovirus immunization, mice were challenged with 5×10^6 pfu of vHCV1-3011. Groups of mice, immunized with RAdNS3 plus rat IgG or plus saline as well as a group of mice injected with anti-CD137 alone were included as controls. Three days after challenge with vHCV1-3011, mice were sacrificed, the ovaries were extracted and vaccinia viral load was determined as described in methods. As it is shown in Fig. 3, immunization with RAdNS3 or with saline was unable to protect mice against challenge. Similarly, administration of anti-CD137 antibodies alone, 6 days before vaccinia challenge did not protect mice against infection. However, immunization with RAdNS3 followed by anti-CD137 administration was able to fully protect mice from challenge (reduction of viremia under the limit of de-

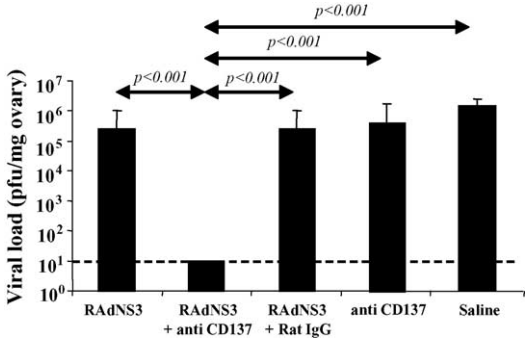

Fig. 3. Anti-CD137 antibody administration after immunization with RAdNS3 confers protection against infection with vHCV1-3011 expressing HCV polyprotein. Groups of 10 mice were immunized with: (i) RAdNS3; (ii) RAdNS3 plus anti-CD137; (iii) RAdNS3 plus rat IgG; (iv) anti-CD137 alone; or (v) with saline: 10 days after immunization mice were challenged i.p. with 5×10^6 pfu of vHCV1-3011. Three days after challenge, ovaries were harvested and vaccinia titre was measured by plating on BSC-1 cells. Viral load is expressed as the log of virus titre per mg of ovary. Bars represent the mean average of virus titres from 10 mice + S.E.M. Doted line represents the limit of detection of vaccinia infection in the BSC-1-based bioassay. Similar results were obtained in two independent experiments.

tection in the BSC-1 cell-based bioassay). Administration of rat IgG or saline after RAdNS3 immunization did not have any protective effect.

4. Discussion

T cell responses against viral antigens are present in chronic HCV carriers. However, they are weak and inefficient [3–10,14,15,45]. In fact, the frequency of CTL precursors against HCV proteins found in patients with chronic hepatitis C is very low, in comparison to that found in HIV or CMV infections [46–48]. It has been described that HCV can infect DC and that expression of HCV proteins inside DC induces an impairment of its stimulatory functions [20] and this impairment might lead an inefficient priming of anti-HCV T cell responses [19,29,49]. Thus, there is a need for the development of strategies able to induce potent immune responses against HCV proteins that could be effective in the treatment and prevention of HCV infection.

Costimulatory signals are essential for full activation and proliferation of activated T cells and it is generally accepted that in the absence of these signals, the T cell response is ineffective and may often lead to death or unresponsiveness of antigen-specific T cells. CD137 molecule has been implicated in providing expansion and survival signals to T cells, and thus, might be a target for strategies aimed at augmenting weak T cell responses. In the present work we have tested the effect of the administration of an agonistic anti-CD137 antibody after immunization with a suboptimal dose of a recombinant adenovirus expressing HCV-NS3 protein (RAdNS3) on the induction of anti-NS3 $CD4^+$ and $CD8^+$ cellular immune responses. We have administered the anti-CD137 antibody 4 days after immunization with the adenovirus because the expression of CD137 molecule on the surface of T cells is induced after antigen driven-TCR activation of T cells, with a peak expression at 48–72 h [50]. After this immunization schedule we found that anti-CD137 administration strongly improved IFN-γ production by $CD4^+$ T cells in response to NS3 protein (Fig. 1). It has been described that the agonistic anti-CD137 antibodies in vivo have a preferential role as a costimulation molecule for $CD8^+$ T cells, with no detectable effect on $CD4^+$ T cells [34–36,51]. Moreover, some experiments have suggested that CD137 stimulation might induce $CD4^+$ T cell anergy [52,53] or activation-induced T cell death [54]. However, there are several reports in in vitro models supporting the costimulatory effect of CD137 on $CD4^+$ T cell proliferation and survival activation. [34,50,54–58]. In addition, recent reports using TCR transgenic mice show a positive role of CD137 in $CD4^+$ T cell activation in vivo [59,60]. Further work in defined in vivo systems of antigen-specific $CD4^+$ T cells is needed to clarify these discrepancies. The recent finding of CD137 on regulatory T cells [61] might hint the research on theses issues. In any case, we have found that anti-CD137 administration following vaccination with RAdNS3 clearly augments $CD4^+$ responses against HCV NS3, and this finding might have important implications for vaccination against HCV infection.

Administration of anti-CD137 antibodies after RAdNS3 injection allowed the induction of a strong CTL response against target cells pulsed with peptide p1215, which encompasses an immunodominant epitope from NS3 in BALB/c. This increase in CTL activity was associated with a marked increase in the production of IFN-γ in response to the peptide. It has been described that in vivo administration of an agonistic anti-CD137 antibody preferentially expands $CD8^+$ T cells that recognise non-dominant epitopes giving rise to broader immune responses (in a phenomenon named $CD8^+$ epitope broadening) [62]. We have tested if the anti CD137 mAb administration was able to expand $CD8^+$ T cells specific for other non-dominant epitopes from NS3 described in our previous publication [17]. However, we have only found an improvement in both CTL and IFN-γ production capacity in response to the immunodominant p1215 and not in response to the other peptides (data not shown).

We tested the capacity of this immunization protocol combining RAdNS3 and anti-CD137 antibody administration to protect mice from the infection of the recombinant replicative vaccinia virus vHCV1-3011 expressing the HCV-polyprotein, as a surrogate for HCV infection. We show in this model that vaccination of mice with a suboptimal dose of RAdNS3 is unable to protect mice from vaccinia challenge. However, when RAdNS3 immunized mice were supplemented with anti-CD137 administration, a full protection against challenge with the recombinant vHCV1-3011 was observed. Our data suggest that anti-CD137 administration after immunization with an antigen will provide a valuable tool for the induction of strong antiviral CD4 and CD8 T cell immune responses. Recombinant adenoviral vectors expressing HCV proteins, combined with anti CD137 administration might be useful, not only in prophylactic vaccination, but also in the active immunotherapy of HCV chronically infected patients.

Acknowledgements

The authors thank Oihana Murillo for her help on anti-CD137 antibody production and purification and to Juan Percaz and Javier Guillen for technical collaboration at the animal facilities. This work was supported in part by grants from Instituto de Salud Carlos III (PI030566) to J.J. Lasarte, from Ministerio de Ciencia y Tecnología (SAF2001-1119) to P. Sarobe, from CICYT (SAF2000-0059) to F. Borrás-Cuesta, from the European Union (QLK2-CT-1999-00356) to J.J. Lasarte and J Ruiz, from CICYT (SAF2002-0327) to J. Prieto, from CICYT (SAF2002-0373) and RETIC C03/10 to I. Melero, and by Instituto de Salud Carlos III (C03/02). This project was funded by “UTE project CIMA”.

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