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Review

DNA vaccine strategies for hepatitis C

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Hepatitis C virus (HCV) is a positive-strand RNA virus that codes for at least six different proteins. Its worldwide prevalence ranges from 0.5 to 10%, and it is currently believed that more than 70% of HCV-infected patients will evolve towards a chronic carrier state, with the risk of developing cirrhosis associated with an enhanced chance of evolution towards hepatocellular carcinomas (HCC). These risks remain difficult to assess due to the wide variety of follow-up items and inclusion criteria that have been used in natural history studies reported thus far (1). In France, HCV infections have become the prevalent cause of HCC, and it is estimated that more than 30 000 cases of HCC directly related to HCV infection will be accounted for by the year 2010 (2). Community-acquired infection is still common, causing a significant amount of morbidity as well as important economic burdens. Only a minority of patients benefit from existing antiviral therapies, thus making highly desirable the development of a preventive, and possibly even more, a therapeutic vaccine.

Several observations from clinical and experimental studies of humans and chimpanzees suggest that failure to mount an effective immune response against HCV is responsible for the high rate of chronicity characterizing infections by this agent. These studies have shown that: 1) following a self-limited infection or during a chronic carrier state, re-infection with a heterologous or homologous inoculum can occur (3,4); 2) infection by multiple genotypes, in particular in high-risk populations such as hemophiliacs or drug abusers, is possible (5); and 3) HCV exists in the infected host as a very complex population of mutant viruses or quasispecies (6). The quasispecies distribution seems to fluctuate with duration of infection and in response to antiviral treatment, and may be responsible for the emergence of immune-escape mutants. Taken together, these data suggest that the natural immunity fails to control the infection, although a certain degree of immunity appears to be mounted following primary infection. Indeed, Prince et al. documented in the chimpanzee model that second episodes of hepatitis following challenge tend to be milder (4). Fig. 1 provides a summary of the main histological features associated with a natural course of HCV infection.

The development of a vaccine against HCV obviously faces multiple challenges. The identification of immunogenic antigens carrying neutralizing determinants has yet to be documented. Preliminary data suggest that the N-terminal part of the envelope glycoprotein E2, the so-called hypervariable region (HVR, 7) could contain a neutralizing epitope (8–10). This region contains several B-cell as well as CTL epitopes, and specific antibodies directed at this region were reported to block viral attachment to susceptible cells (11). Due to the lack of a sensitive *in vitro* tissue culture assay for HCV, it has not yet been possible to test the neutralizing potential *per se* of such candidate antibodies, let alone their cross-neutralizing ability. An active search is under way to develop substitute assays, and a group has recently developed a specific cell-binding assay whereby the purified glycoprotein E2 is quantitatively absorbed onto susceptible cells (12). Antibodies capable of neutralizing binding of E2 in this

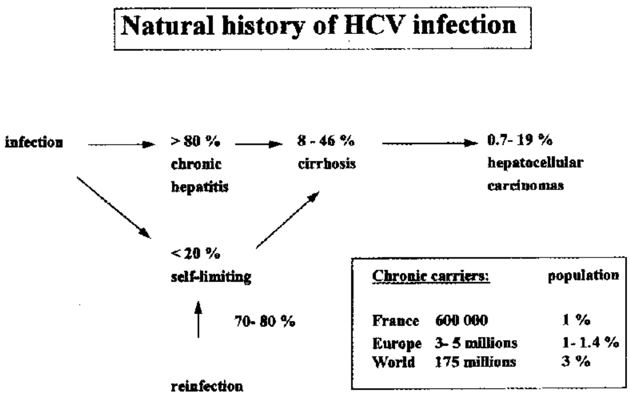

Fig. 1. Histological diagnosis associated with the natural course of HCV infection and prevalence in France, Europe and worldwide (2,44).

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assay (neutralizing of binding antibodies or NOB antibodies) have been detected in up to 40% of chronically infected patients. In addition, protection of chimpanzees immunized with E1 and E2 was correlated not only with high anti-E2 antibody titers but also with high titers of NOB antibodies (12,13).

Identification of components of the cellular immune machinery (such as CD8+ cytotoxic T lymphocytes (CTL) and CD4+ T-helper lymphocytes) capable of eliminating or facilitating the elimination of the virus are currently the focus of active investigations. Circulating blood lymphocytes as well as liver infiltrating lymphocytes have been successfully used to identify a number of CTL determinants (for review, see (14)). These have been mapped to virtually all encoded HCV antigens. At the present time no correlation has been established between CTL activity against any particular determinant or antigen and development or outcome of disease. A favorable response to interferon treatment appears nonetheless to be associated with enhanced CTL activity. In earlier studies, an enhanced (CD4+) proliferative response directed at the nucleocapsid (C) and the NS4 protein, had been found in association with self-limited or asymptomatic evolving infections (15,16). More recent studies have documented that CD4+ type responses directed not only at these two antigens but also at the glycoproteins E1 and E2 and the non-structural proteins NS3 and NS5 were associated with control of infection and/or better response to antiviral treatments (16).

Both humoral and cellular immune responses are likely to be important for controlling HCV infection. Nucleic acid-based immunizations have been shown to elicit both types of responses which, in several viral models, have been able to confer protection (17–20). The most commonly used routes of DNA delivery are the intramuscular (i.m.) and the intraepidermal (i.e.) injection routes. Immune responses generated by these two delivery routes appear to involve different mechanisms. For example, Torres et al. have shown that transfected epidermal cells, but not injected muscle cells, play a central role in DNA-initated immune responses, suggesting that the latter have a minimal function in the presentation of expressed peptides (21). The goal of this review is to present the latest work reported in the field of gene immunization against HCV and discuss its potentialities for the development of both a preventive and therapeutic vaccine.

Injection of Plasmid DNA for the Induction of HCV Anti-E1 and Anti-E2 Antibodies

It is not obvious how to exploit the participation of immune responses directed at the HCV glycoproteins E1 and E2 for the development of a vaccine in view of accumulated data demonstrating that both antigenic domains, but particularly E2, are prone to mutational changes and the appearance of immune escape mutants (7,9,22). Nonetheless, recent published data have shed new light on the role of anti-E1 and anti-E2 antibodies, in both the human and the chimpanzee models. It is now clear that antibodies against the E2 protein are widely cross-reactive, in spite of the existence of hypervariable regions within the coding sequence (13,23,24). There seem to be at least two types of anti-E2 neutralizing antibodies: those that are directed at the hypervariable region 1 (HVR1) and that could play a role in the control of acute infection (11), and those that would be directed at more conformational determinants and possibly involved in the control of chronic infection (25). High anti-E1 antibodies at initiation of interferon treatment appear to correlate with long-term response (Maertens, personal communication) while induction of anti-E1 antibodies in chronically infected chimpanzees would participate in the normalization of the histological status of the animals (Maertens personal communication).

Four studies have reported the successful induction of humoral responses following the direct injection of E2 encoding plasmids in mice (26–28). These studies addressed issues related to the induction of anti-E2 humoral immune responses in terms of: 1) evaluating the competency of DNA immunization for induction of high and stable anti-E2 antibody titers; 2) identifying E2 subtype variable or conserved immunogenic regions; 3) analyzing the capacity of E2-plasmid induced antibodies to recognize a mature form of the protein as a predictor of neutralization.

In their study, Tedeschi et al. (26) used a plasmid expressing the near full-length HCV E2 sequence under the CMV promoter and were able to document seroconversion to anti-E2 antibodies as early as week 2 p.i. following i.m. injection of the DNA. Presence of plasmid DNA in the mice quadriceps was demonstrated up to 4 months p.i., confirming previous studies that have documented long-term persistence of the injected DNA in the injected tissues (19). Immune reactivity of the induced sera was assessed using a panoply of synthetic peptides and two linear epitopes were successfully mapped. In a more recent work, Nakano et al. (27) compared the induction of humoral immune responses generated after injection of a similar construct as well as a panoply of HBsAg-expressing plasmids carrying distinct, non-overlapping E2 putative antigenic domains. The latter insert sequences were cloned in fusion with the HBsAg sequences, in replacement of the pre-S2 sequence. None of these constructs appeared to efficiently secrete the expressed antigens. Those were systematically located within the cytoplasms of transfected cells. All constructs were injected i.m. or i.e. in BALB/c mice to allow for a fine comparison of induced responses in correlation with the injection routes. Typical doses of 100 μg and 5 μg of gold-coated DNA per mouse were injected, respectively, i.m. and i.e., and typically one booster injection was performed at 5 weeks p.i. Table 1 illustrates the results obtained for various E2-encoding plasmids: pCIE2t, expressing the near full-length coding region of E2 (aa 384–674) and a panoply of plasmids, pS2SE2A-E expressing contiguous 60 amino acids of the coding domain of E2 in fusion with the HBsAg. Important differences were seen between the two injection modes for all plasmids, in terms of seroconversion rates and antibody titers, while isotypes of induced antibodies were identical, IgG2a and IgG2b (IgG1 was never detected). As shown in Table 1, differences in antibody titers were particularly dramatic for the pS2.SE2C plasmid, as i.e. induced antibody titers were 100-fold higher than i.m. induced at week 12 p.i. Because i.e. injections target plasmid DNA in a tissue, the epidermis, particularly rich in Langerhans cells (cells that play an important role in antigen presentation and immunocompetence of the skin, (28,29)), it has been speculated that such an "environment" could explain why i.e. injections are generally more potent at inducing strong immune responses. In Nakano's study, more intriguing was the observation that different determinants (linear epitopes) seem to be recognized depending on the injection mode of the plasmids DNA. Indeed, Western blot analysis and peptide-based mapping revealed that, for both pS2.SE2C- and pCIE2t-induced sera, different epitopes were recognized by the induced antibodies, independent of their titers (30). In addition, cross-reactivity of the induced sera as tested against purified E2 proteins as well as synthetic peptides derived from two distinct viral subtypes was also found to be dependent on the injection mode of the plasmids. These data suggest that not only quantitative but also qualitative differences in the induced immune responses can be observed that are linked to both the nature of the antigen expressed and the immunization route. It is interesting to note that similar observations have recently been described in the influenza model (31). In this study, authors report that the viral nucleoprotein-specific immunoglobulin subclass and cytokine responses elicited by DNA vaccination are dependent on the injection route of the DNA. While endpoint immunoglobulin

TABLE 1

Anti-E2 antibody response in seroconverted mice: maximal, mean titers and isotypes. Groups of 4 mice were immunized either i.m. or i.e. (with a gene gun) with the indicated plamids and anti-E2 antibody titers and isotypes were evaluated at 6, 12 and 18 weeks post-inoculation (p.i.). Numbers in bold face type represent maximal antibody titers, while number in parentheses represent median titers. Adapted from Nakano et al. (27)

Plasmids	Injection mode	Titers			Isotypes
		6	12	18	15 (weeks p.i.)
pS2.SE2A	i.m.	**9336** (9336)	**6892** (1815)	**1620** (166)	IgG2a, IgG2b
	i.e.	**59** (54)	**8280** (2832)	**12 946** (3304)	
pS2.SE2B	i.m.	– (–)	**20** (20)	**161** (72)	
	i.e.	**104** (76)	**194** (118)	**127** (72)	
pS2.SE2C	i.m.	**390** (390)	**300** (170)	**99** (90)	IgG2a
	i.e.	**3700** (1862)	**>43 700** (>15 134)	**35 509** (13 337)	IgG2a, IgG2b
pS2.SE2D	i.m.	– (–)	**20** (20)	**53** (<37)	
	i.e.	**50** (48)	**55** (<40)	**240** (101)	
pS2.SE2E	i.m.	**20** (20)	**20** (20)	**51** (<36)	
	i.e.	– (–)	**32** (32)	**59** (50)	
pCIE2t	i.m.	**1288** (761)	**>43 700** (>15 899)	**31 590** (11 410)	IgG2a, IgG2b
	i.e.	**3637** (977)	**>43 700** (>21 807)	**>43 700** (>24 904)	IgG2a, IgG2b

IgG titers were identical, muscle injection resulted in predominantly IgG2a responses, and gene immunization yielded a preponderance of IgG1 antibodies. In addition, *in vitro* production of cytokines following specific recall T-cell activation was also quite different, as for example Il4 production could never be detected following i.m. injection but increasing concentrations were observed following i.e. injection of the plasmids. Because the choice of the injection route can modulate the immune responses induced, Fournillier et al. have recently performed a systematic analysis of the i.m. and i.e. injection routes used either alone ("single delivery route") or in combination ("combined delivery route") for the induction of anti-E2 antibodies in BALB/c mice (28). While aspects of the immune response (seroconversion rates, antibody titers and epitope specificity) were influenced by either or both of the type of antigen expressed and the route of injection, others (antibody isotypes and cytokine profiles) were not. The simultaneous combination of delivery routes did represent the most favorable way to induce a broad immune response as antibodies generated in such protocols recognized the greatest number of peptide-epitopes tested in the study. These observations are in the process of being confirmed in a small primate model (Fournillier et al., personnel communication).

While it has been possible to document induction of humoral responses to the HCV-E2 protein following direct injection of plasmid DNA, induction of cellular responses remains to be described. We have observed in preliminary experiments that i.m. injection of E2-encoding plasmids is indeed associated with the splenic production of INFγ but not Il4, and that the levels of cytokines produced are low but constant over time, at least as observed between 3 and 24 weeks p.i. (Nakano, unpublished observations, 28). Induction of CTL following E2-derived plasmid injections has been difficult to observe. Splenic-derived CTL were identified by Saito et al., but were particularly weak (at most 20% specific lysis for E:T ratios ranging from 1:100 to 1:200, 32). Thus while E2-DNA immunogens may be efficient at inducing antibodies, the neutralizing capacity of these antibodies remains to be documented and induction E2 specific cellular immune responses obviously need to be optimized. Very similar observations have been made for E1-derived plasmids (32). Besides being poor inducers of cellular responses, El-plasmids are also associated with a low, genotype-restricted antibody response (Fournillier et al., unpublished observations). Taken together, the data gathered by the different groups working in the field so far point to the probable need to combine different types of immunogens (naked DNA and recombinant protein, for example) for an optimized immune response targeted at both HCV E1 and E2.

Plasmids Encoding the Viral Nucleocapsid: Direct Injection and Induced Immune Responses

HCV nucleocapsid (CAP) may represent a valuable component in the development of a vaccine, as it is the most conserved viral antigen (about 95% homology at the amino acid level has been described between the different viral genotypes/subtypes). In addition, internal antigens such as nucleocapsids have been shown in various models (e.g. rabies virus, hepatitis B virus and influenza virus) to be associated with protective immune responses, most likely via the generation of effective CTL (17,33,34). Different strategies have been described for the design of HCV CAP-expressing plasmids. These have included the use of plasmids containing the full-length CAP sequence under the control of a CMV promoter (35–37) as well as plasmids containing truncated sequences of CAP expressed in fusion with the hepatitis B surface antigen (HBsAg, 35,38,39). HBsAg-derived hybrid proteins have been shown in different models to be particularly effective

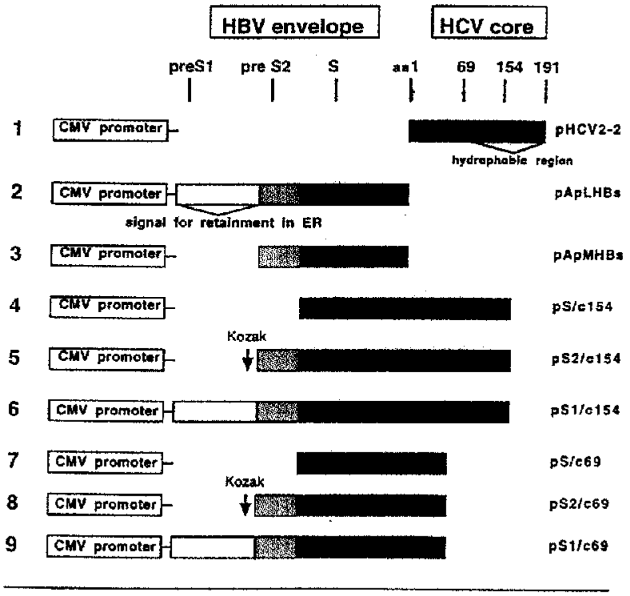

Fig. 2. Stategies used by Geissler et al. for the construction of core-expressing plasmids. The pHCV2-3 construct encodes the "wild" type 21 Kda HCV core protein. The pApMHBs and pApLHBs constructs include the preS2/S and preS1/preS2/S genes, encode the "wild" type MHBs and LHBs proteins, respectively. Constructs 4–9 encode for the various HBV envelope-HCV core chimeric proteins. Adapted from Geissler et al. (39).

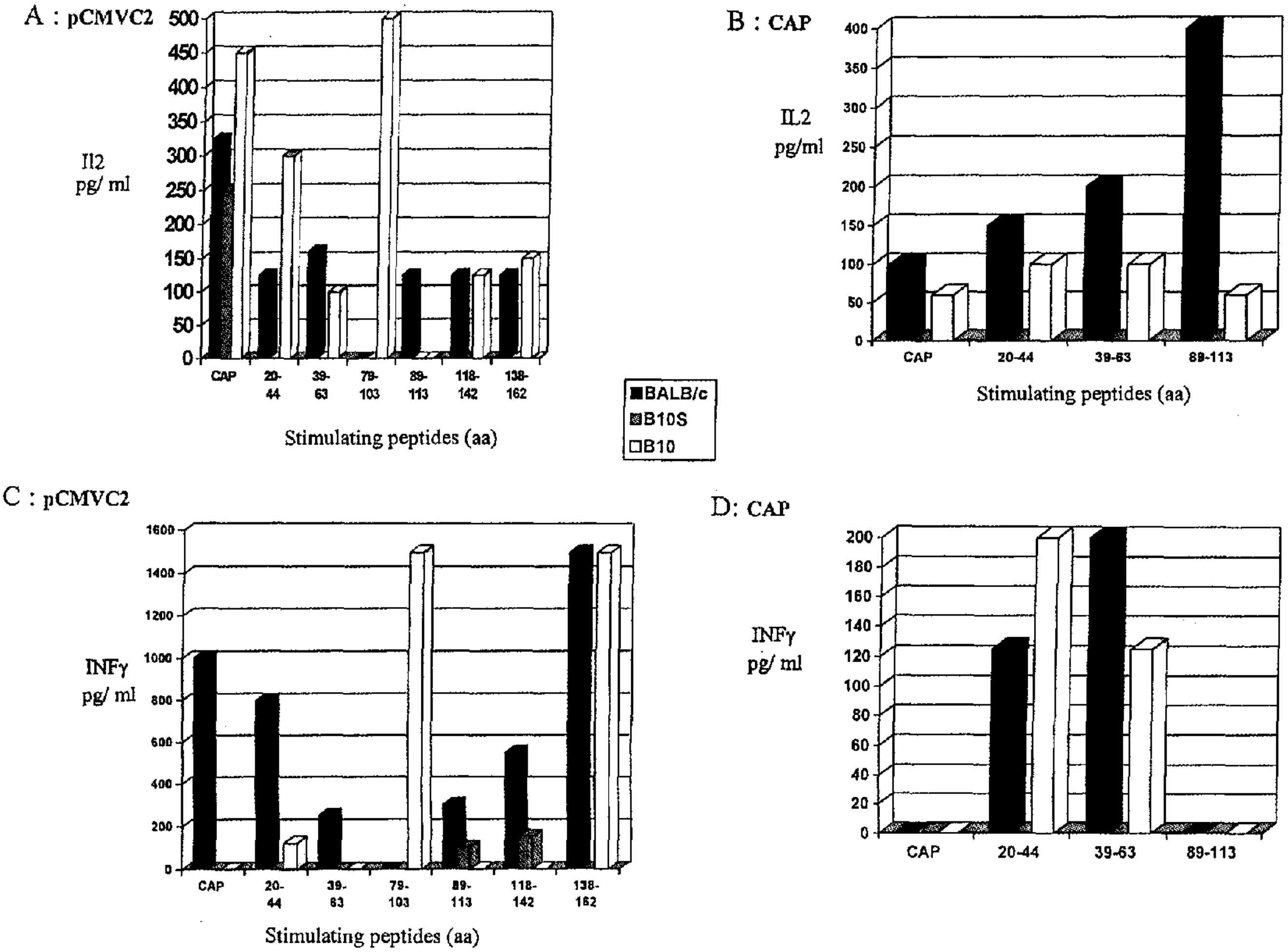

Fig. 3. Comparison of CAP-specific cytokine production by BALB/c, B 10 and B 10S splenic T cells following genetic or protein-based immunization. Groups of three mice were immunized with the pCMVC2 vector (expressing the full-length CAP gene) or purified CAP antigen. Splenic cells were cultured in the presence of the various indicated stimulating peptides used at three different concentrations (0.25, 2.5 and 6 mg/ml). Production of Il2 following immunization with pCMVC2 (A) or with CAP (B) and production of IFNγ following immunization with pCMVC2 (C) and CAP (D) were recorded and expressed in pg/ml. Results given are representative values from two separate experiments. Only those peptides that resulted in cytokine production are indicated. From Koshy & Inchauspé (14).

for the presentation of foreign epitopes in immunization experiments using *in vitro* produced particles (40,41).

HBV-HCV chimeras have been constructed, either by insertion of HCV sequences in place of or within the coding sequence for the preS2 gene (35,38) or downstream from the S gene (39). Fig. 2 is an illustration of the different HCV-core-encoding plasmids recently described by Geissler et al. (39). Up to nine different plasmids were compared in this study. *In vitro* experiments demonstrated expression of both HCV and HBV antigens from these plasmids in the cytoplasms of transiently transfected cells. Secretion of the viral antigens in supernatants of transfected cells could never be documented in spite of the use of several different monoclonal antibody sandwich RIAs, thus suggesting that most of the expressed antigens remained as intracellular forms. Seroconversion rates and antibody titers were systematically found to be lower with plasmids expressing the HCV-CAP directly under the control of the CMV promoter (i.e. from a non-chimeric plasmid), while up to 60–100% rates and at least 1 log increase in antibody titers could be observed in mice injected with HBV-HCV chimera independent of the HCV insert. Proliferative responses (followed by the incorporation of ^{3}H Thymidine and *in vitro* splenic production of cytokines) were mainly associated with one specific chimeric construct (encoding core aa 1–154, pS2/c154). Up to a 5-fold increase in incorporated ^{3}H

Thymidine could be observed when compared with a plasmid encoding the HCV core sequence directly under the CMV promoter (pHCV2–2). The chimeric construct also yielded CTL activity comparable to that detected after injection of the pHCV2–3 plasmid, while other chimeras resulted in lack of CTL induction. Thus the choice of the expressing nucleotide sequence can directly impact on the immune responses induced.

Inchauspé et al., using both chimeric and non-chimeric CAP-expressing plasmids, performed comparative experiments in three different mouse haplotypes H-2^d, H-2^b and H-2^s (38). While dramatic differences in seroconversion rates were observed between the different lines of mice for all immunogens, HBV-HCV chimeric plasmids were the most efficient at inducing anti-CAP antibodies independent of the haplotype of the mice. The fastest seroconversion rate to anti-CAP antibodies was observed in BALB/c mice (H-2^d). A detailed analysis of antigen-specific *in vitro* cytokine production by the splenic cells of plasmid-injected mice revealed the presence of numerous potential T-helper epitopes, some of them apparently being unique to specific MHC backgrounds as described below. Overall, induction of proliferative responses (CD4+) following the direct injection of CAP-encoding plasmids was demonstrated in proliferation assays as well as in analysis of *in vitro* cytokine production following stimulation with purified CAP or specific synthetic peptides (36,38). Both interferon gamma (IFNγ) and interleukin 2 (Il2) could be detected (concentrations ranged from 30 to 1500 pg/ml), while Il4 and Il10 were not detectable or detectable at very low levels only (<30 pg/ml). Such profiles were detected whether CAP was expressed as a fusion or a nonfusion with the HBsAg, and were comparable in different haplotypes of mice. In contrast, cultures of spleen cells from mice immunized with a highly purified CAP protein, in absence of adjuvant, gave rise to the production of Il2, Il4, Il10 and IFNγ ranging from 30 to 280 pg/ml. In these experiments, *in vitro* stimulation was performed using purified CAP as well as CAP-specific overlapping synthetic peptides. Putative T-helper epitopes could be mapped and, overall, the same peptides had identical effect on cells taken from DNA- or protein-injected animals, with the exception of one peptide (aa. 79–103) that elicited a response in DNA, but not protein-immunized mice (see Fig. 3, 38).

In vitro and *in vivo* detection of specific (CD8+) CTL was reported in different studies following intramuscular i.m. or i.e. injection of CAP-derived plasmids (36,37,39,40, Vidalin personal communication). They illustrate the following points: 1) induction of CAP-specific CTL following DNA injection does not appear very efficient (maximal of 20% specific lysis for an effector to target (E:T) ratio of 1:100). In comparison, for example, the CTL-specific lysis following one single injection of HBsAg encoding plasmid DNA was typically found to be 80% for an E:T ratio of 1:20 (Vidalin, personal communication); 2) such a low level of detection is independent of the time of analysis post-immunization (p.i) and was found to be identical at 5 and 24 weeks p.i. (O. Vidalin, personal communication); 3) in a mouse tumor rejection model, CAP-specific CTL were nonetheless apparently capable of eliminating CAP-expressing tumors when compared to appropriate controls (37). In this latter model, rejection of tumors was observed within a short period of follow-up post challenge (20 days) suggesting that CTL were efficiently primed *in vivo*. Up to 3 booster injections (using 100 μg of DNA per injection per mouse) were nonetheless necessary to achieve this rejection. Although somewhat artificial, this type of *in vivo* model may provide alternative ways to assess the induction of DNA-induced specific HCV CTL as small animal models are not available. We have observed that, using a synthetic peptide corresponding to a previously described murine CTL epitope (41), it was possible to detect up to 80% specific lysis following i.m. or i.e. injections of CAP-expressing plasmid DNA.

We have recently performed comparative analysis of anti-CAP immune responses induced following i.m. or i.e. injection, using a Gene Gun, of CAP-expressing plasmids. Overall, anti-CAP seroconversion rates and antibody titers were found to be slightly higher following i.e. injections rather than i.m., but no differences could be observed at the level of CTL induction. Thus, changing the injection route of HCV CAP-plasmid DNA does not appear to enhance cellular immune responses to the antigen. Other strategies would have to be devised to achieve this goal, such as, for example, co-injection of cytokine encoding plasmid DNA. Preliminary results obtained by Geissler et al. (39) who co-injected GM-CSF, Il2 and Il4 encoding plasmids with HCV CAP-plasmids showed that this approach could result in a 2- to 3-fold increase in anti-CAP antibody titers or seroconversion rates, but did not produce any improvement on the induced CTL. All of the above studies have been performed in a single mouse haplotype (BALB/c, H-2^d). It would be of obvious interest to test the different HCV-plasmids in other murine haplotypes as well as other animal species. Our laboratory is in the process of doing so, in particular using transgenic mice for human HLAs. Such data will be important in the design and testing of optimal plasmid constructs and/or for the evaluation of combinations of immunogens or adjuvant.

Towards a Nucleic Acid-Based Vaccine Against HCV?

As reviewed above, examples of studies focused on the evaluation of DNA-based immunization for the induction of immune responses specific for HCV antigens are still scarce. Additional experiments are ongoing in different laboratories that focus on other HCV antigens, including the non-structural protein NS3 that has been shown to induce strong CD4+ and CD8+ immune responses. Among the many obstacles that the development of an efficient HCV vaccine will face, are the lack of appropriate analytical tools. These include: 1) the lack of an *in vitro* replication assay; 2) the lack of a small susceptible animal model beside the chimpanzee and; 3) the lack of purified antigens, in particular the viral glycoproteins. HCV E1 and E2 antigens have been reported to associate and form two types of complex heterodimers: covalently and non-covalently linked, the latter presumably mimicking the native structure believed to exist on the surface of virions (42,43). Assessing the relevance of anti-envelope antibodies and their potential for neutralization, while awaiting appropriate neutralization assays, could involve immunoprecipitation studies using such complexes as a potential marker for neutralizing capacity. Much basic work remains to be performed to evaluate the benefit of combined immunization protocols (e.g. DNA priming followed by protein boosting) for induction of either a stronger humoral or an enhanced cellular response. As described in various published reports, combining DNA immunogens with other types of immunogens yields immune responses that are strongly dependent on the antigen used and, basically, empirically exhaustive approaches must be tested on a case-by-case basis.

Finally, clinical studies have still to document the types of immune responses that are beneficial in the control and/or prevention of HCV infections. Such studies, as reviewed in the introduction of this chapter, seem to point towards the benefit of an early, strong and broad cellular response that appears to be associated with either spontaneous viral clearance or better outcome of antiviral treatments. Recent data, in addition, suggest that NOB antibodies may also be important for clearance of virus. Abrigiani et al. have indeed documented, in long-term follow-up studies (up to 8 years), that spontaneous recovery from infection appears to be linked to the appearance of high anti-NOB antibody titers (25). Both fundamental and clinical studies are warranted for the design of the most efficient strategies for the development of a HCV vaccine, including a gene-based vaccine.

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