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Immunization with recombinant protein: conditions for cytotoxic T cell and/or antibody induction

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Abstract. Safe vaccines should optimally induce both cell-mediated and humoral immunity. Recently, it has been shown that protective cytotoxic T cells (CTLs) can be induced not only with live vaccines, but also with recombinant viral proteins. This report shows in C57BL/6 (H-2^b) mice that the recombinant nucleoprotein (N) of vesicular stomatitis virus (VSV) induced protective CTLs but no neutralizing antibodies in mice, whereas the recombinant glycoprotein (G) of VSV alone induced neutralizing antibodies but no CTLs. If the N and G of VSV were coinjected, both CTLs and a long-lasting neutralizing IgG response was measurable, demonstrating that mixed vaccines can be used to induce protective CTLs and antibodies with an efficiency comparable to live virus. In an attempt to define optimal conditions for CTL priming, the intravenous, intraperitoneal and subcutanous route of injection were compared. Intravenous injection of recombinant VSV-N induced up to 30 times higher responses than the latter two routes. Finally, we tried to define conditions inducing only CTLs and no antibodies binding to the native protein form, or vice versa, only antibodies and no CTLs. Intravenous injection of boiled VSV-N induced a CTL response but no antibodies specific for the native VSV-N, whereas VSV-N injected subcutanously in incomplete Freund's adjuvant induced high amounts of anti-VSV-N antibodies but virtually no CTLs. The conditions defined here permit vaccines to be designed which would function along selected and defined immunological effector pathways.

Introduction

Optimal vaccines usually induce an efficient antibody response together with cytotoxic T cells (CTLs). Dependent upon the infectious agent generation of either antibodies or CTLs alone may under selected conditions cause severe immunopathological complications. For example, antibodies in the absence of CTLs have been shown to cause immunopathology in the case of Dengue fever (Halstead 1988;

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Halstead 1988), feline infectious peritonitis (Vennema et al. 1990; Corapi et al. 1992), lymphocytic chorimeningitis virus (LCMV) (Battegay et al. 1993), measles virus (Fulginiti et al. 1967), and respiratory syncytial virus (Kapikian et al. 1969; Prince et al. 1986; Connors et al. 1992). On the other hand and under rare conditions, CTLs alone have been shown to enhance immunopathology and death in the case of LCMV (Oehen et al. 1991). Vaccines derived from killed viruses usually do not induce CTLs but trigger antibody responses (Blanden 1974; Bachmann et al. 1993b). In contrast, attenuated, live viruses which still replicate fully or abortively in the host (e.g., UV-light inactivated viruses), usually induce excellent and long-lasting immune responses, comprising both CTLs and antibodies (Fields 1985; Bachmann et al. 1994a). However, a disadvantage of live vaccines is that they are safe in healthy individuals but may cause complications in immunosuppressed individuals (Horstmann 1979; Salk and Salk 1977; Nkowane et al. 1987), including AIDS patients. Therefore, vaccines consisting of non-replicating material that still induce CTLs are sought. Recently, we have shown that recombinant viral proteins derived from vesicular stomatitis virus (VSV) and LCMV are able to induce potent and protective CTL responses in mice (Bachmann et al. 1994b). Similar observations have been made for other model antigens such as ovalbumin (Rock et al. 1990; Fenton et al. 1993), the SV40 large T antigen (Schirmbeck et al. 1992) or hepatitis S antigen (Schirmbeck et al. 1994a, b).

The nucleoprotein (N) of VSV, which belongs to the family fo *Rhabdoviridae* (Wagner 1987), is used here as a model antigen to induce, in an MHC-restricted fashion, protective CTLs but not neutralizing antibodies, whereas the glycoprotein is used to induce protective neutralizing antibodies but no CTLs. In mice, VSV induces both a CTL and a neutralizing antibody response (Kelley et al. 1992; Rosenthal and Zinkernagel, 1980). The antibody response is characterized by an early T help-independent IgM response peaking around day 4 (Charan and Zinkernagel 1986; Bachmann et al. 1993a), which is followed by a strictly T help-dependent IgG response by day 6 to 8 (Leist et al. 1987; Bachmann et al. 1993b). It is the glycoprotein of VSV (G) which induces neutralizing antibodies (Kelley et al. 1972), whereas the nucleoprotein is the only viral component inducing CTLs in H-2^b mice (Yewdell et al. 1986; Kündig et al. 1993). Using different protein mixtures, routes of injection, adjuvants and denaturing conditions, we here describe ways to induce defined immune responses comprising either CTLs alone, antibodies alone or CTLs and antibodies together.

Materials and methods

Mice

Inbred C57BL/6 (H-2^b) mice were obtained from the breeding colony of the Institut für Zuchthygiene, Tierspital Zürich, Switzerland. Mice were between 8 and 12 weeks of age.

Viruses

VSV-Indiana (VSV-IND) (Mudd-Summers isolate) seeds had originally been obtained from Dr. D. Kolakofsky, University of Geneva, and were grown on BHK 21 cells infected with low multiplicity of infection and plaqued on Vero cells (McCaren et al. 1959). The recombinant vaccinia virus expressing the VSV-N was a generous gift of Dr. B. Moss, Laboratory of Viral Dis-

eases, National Institute of Health, Bethesda. Recombinant virus was grown and plaqued on BSC40 cells (Mackett et al. 1985; Moss and Flexner 1987).

The recombinant baculoviruses expressing VSV-N or -G were a generous gift of Dr. H. L. Bishop, NERC Institute of Virology, UK. They were derived from nuclear polyhedrosis virus and were grown at 28 °C in *Spodoptera frugiperda* (SF9) cells in spinner cultures in TC-100 medium (Matsuura et al. 1987; Li et al. 1993).

Production of recombinant VSV-N and VSV-G

To produce VSV-N and -G, SF9 cells at a density of 2×10^6 cell/ml in spinner flasks were infected with recombinant baculovirus expressing the N or G of VSV with a multiplicity of infection of 10 for 24–48 h at 28 °C. Infected cells were harvested, disrupted by sonication and stored at –20 °C. The presence of N or G was confirmed by Western blot analysis and protein concentrations were estimated from the number of infected cells (Matsuura et al. 1987; Li et al. 1993). Recombinant proteins were diluted and sonicated before immunization.

Serum neutralization test

The sera were prediluted 40-fold in supplemented minimal essential medium, then heat-inactivated for 30 min at 56 °C. Serial twofold dilutions were mixed with equal volumes of virus diluted to contain 500 pfu/ml. The mixture was incubated for 90 min at 37 °C in an atmosphere with 5% CO_2. Serum virus mixture (100 µl) was transferred onto Vero cell monolayers in 96-well plates and incubated for 1 h at 37 °C. The monolayers were then overlaid with 100 µl DMEM containing 1% methyl cellulose. After incubation for 24 h at 37 °C the overlay was flicked off and the monolayer was fixed and stained with 0.5% cristal violet. The highest dilution of serum that reduced the number of plaques by 50% was taken as the titer. Due to the addition of an equal volume of virus, the titer of serum was considered to be one step higher. To determine IgG titers,undiluted serum was pretreated with an equal volume of 0.1 M 2-mercaptoethanol in saline (Scott and Gershon 1970). Neutralizing titers are indicated as $-\log_2$ of 40-fold prediluted sera.

Enzyme-linked immunosorbent assay

The VSV-N-specific ELISA was performed as described (Kündig et al. 1993), using purified VSV-IND to coat ELISA plates (PetraPlastic, Switzerland).

Detection of cytotoxic T cells in vitro

Mice were immunized and spleens were removed 10–12 days later and CTLs were restimulated before testing: 3×10^6 spleen cells from infected mice were cultivated for 5 days in the presence of 5×10^4 irradiated (3000 rad γ) EL4 cells transfected with the VSV-N (Puddington et al. 1986). Cells from two wells were pooled and taken up in 1 ml of medium. Serial three-fold dilutions were made (indicated as dilution of standard culture) and tested in a conventional 5-h ^{51}Cr-release assay on EL4 cells transfected with the VSV-N or with a control plasmid (Puddington et al. 1986).

Detection of cytotoxic T cells in vivo

To determine VSV-N-specific CTLs in vivo, primed mice were challenged intraperitoneally (i.p.) with 2×10^6 pfu of recombinant vaccinia virus expressing VSV-N. Five days later, ovaries were removed, homogenated, sonicated and plaqued on BSC40 cells. Titers are indicated as $\log_{10}$ of virus per both ovaries of an individual mouse (Binder and Kündig 1991).

Results

Protein mixtures allow the simultanous induction of CTLs and neutralizing antibodies

The VSV-N yields the only epitope recognized by CTLs in C57BL/6 (H-$2^{b)}$ mice (Kündig et al. 1993). In contrast, the VSV-G exhibits the single virus neutralizing epitope (Kelley et al. 1972). As expected, intravenous (i.v.) injection of recombinant VSV-N (10 μg) alone induced CTLs only and no neutralizing antibodies, whereas i.v. injection of recombinant VSV-G (10 μg) alone induced neutralizing antibodies, but no CTLs. If, however, these components were mixed before injection, both CTLs and neutralizing IgG antibodies were generated, almost as efficiently as after immunization with live virus (Fig. 1). To determine, whether these CTLs were protective in vivo, a protection assay was performed. Since in vivo replication of VSV is not controlled by CTLs but rather by neutralizing antibodies (Lefrancois 1984; Bachmann et al. 1994c), protection cannot be tested against VSV itself. However, it has been shown that peripheral replication of recombinant vaccinia viruses expressing VSV-N is controlled by primed VSV-N-specific CTLs in H-2^b mice (Binder and Kündig 1991; Kündig et al. 1993). Thus, female mice were immunized with 10 μg of recombinant VSV-N alone, recombinant VSV-G alone, recombinant VSV-N mixed with recombinant VSV-G or live VSV (2×10^6 pfu). After 10 days, mice were challenged i.p. with recombinant vaccinia virus expressing VSV-N (2×10^6 pfu; Table 1). Mice primed with recombinant VSV-G alone or left unprimed had high titers of recombinant vaccinia virus in ovaries 5 days after the challenge. In contrast, mice immunized with recombinant VSV-N alone, recombinant VSV-N together with recombinant VSV-G or with live VSV had eliminated the recombinant virus below detection level by day 5, demonstrating the presence of effective, recombinant VSV-N-specific CTLs. Taken together, these result indicate that combined vaccines consisting of mixed recombinant proteins may induce immune responses as efficiently as live vaccines.

Table 1. Induction of protective cytotoxic T cells with mixed recombinant vaccines. Mice were immunized with 10 μg of recombinant nucleoprotein of vesicular stomatitis virus (VSV-N), glycoprotein of VSV (VSV-G), VSV-N mixed with VSV-G, 2×10^6 pfu VSV wild type (wt) or left untreated. Twelve days later, mice were challenged i.p. with recombinant vaccinia virus expressing VSV-N (2×10^6 pfu). Five days later, ovaries were removed and viral titers were determined as described previously (Binder and Kündig 1991)

Mice immunized with	Recombinant vaccinia virus titer in ovaries ($\log_{10} \pm$ s.e.m.)
Recombinant VSV-N	< 1
Recombinant VSV-G	5.2 ± 0.6
Recombinant VSV-N + VSV-G	< 1
VSV wt	< 1
Unimmunized	5.3 ± 0.7

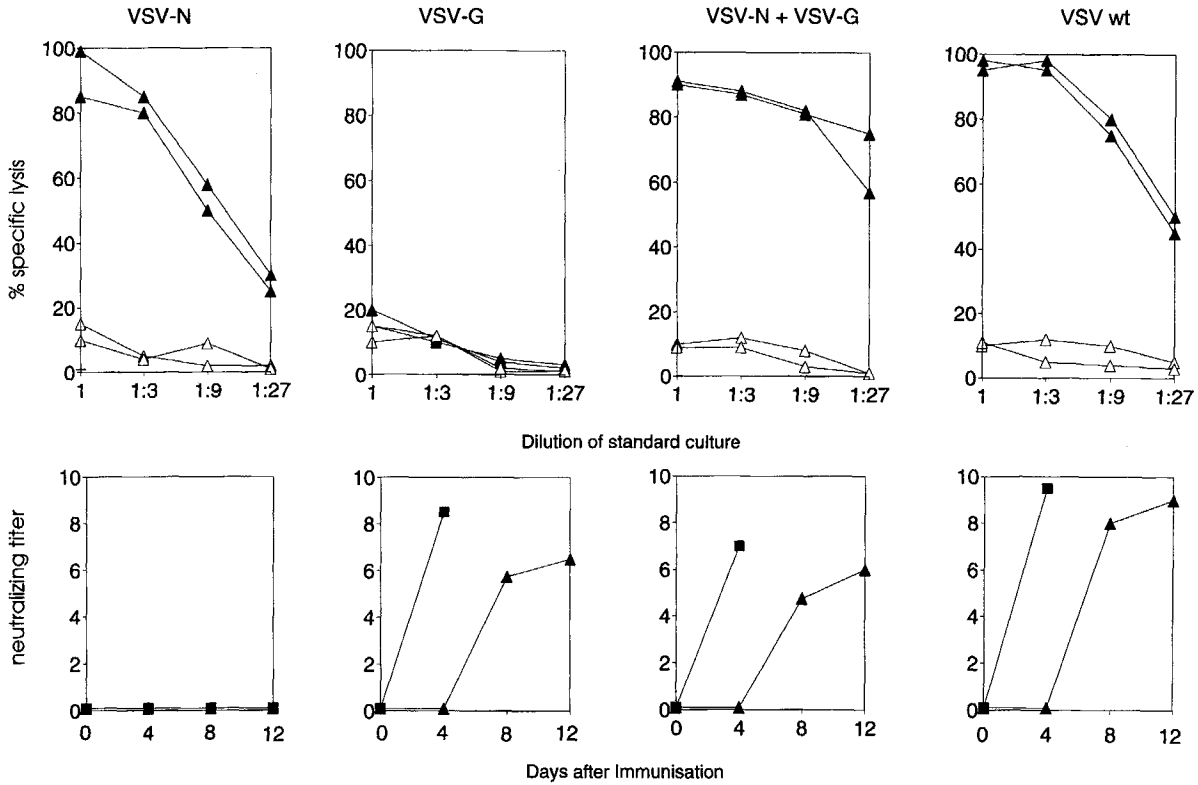

Fig. 1. Mixed vaccines induce immune responses with similar efficiency as live virus. C57BL/6 mice were immunized with 10 μg of recombinant nucleoprotein of vesicular stomatitis virus (VSV-N), glycoprotein of VSV (VSV-G), VSV-N mixed with VSV-G or 2×10^6 pfu VSV wild type (wt). Twelve days later, spleens were removed and restimulated for 5 days with EL4 cells transfected to express the N of VSV and tested in a standard ^{51}Cr-release assay on EL4 cells transfected to express the VSV-N (*close triangles*) or mock-transfected (*open triangles*) (Puddington et al. 1986) (*upper panels*). *Each line* represents an individual mouse. Alternatively, blood was taken at the indicated time points and neutralizing IgM (*squares*) and IgG (*triangles*) titers determined (*lower panels*). The average of three mice is shown; variations were smaller than two dilution steps. One representative of three experiments is shown

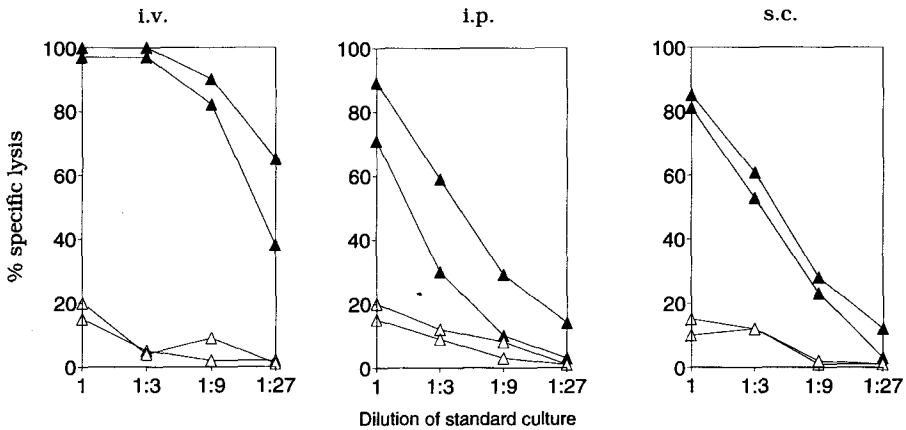

Fig. 2. Effect of the route of immunization on the cytotoxic T cell (CTL) response. Mice were immunized with 10 μg of recombinant VSV-N either i.v., i.p., or s.c. Twelve days later, spleens were removed and restimulated for 5 days with EL4 cells transfected to express VSV-N and tested in a standard ^{51}Cr-release assay on EL4 cells transfected to expresss VSV-N (*closed triangles*) or mock -transfected (*open triangles*) (Puddington et al. 1986). Each line represents an individual mouse. One of two similar experiments is shown

The influence of the route of immunization on the CTL response

To determine the optimal route of immunization, mice were primed i.v., i.p. or subcutanously (s.c.) with 10 μg recombinant VSV-N. Mice primed i.v. showed excellent CTL responses as assessed by ^{51}Cr release. In contrast, mice primed i.p. or s.c. generated only limited CTL responses which were reduced about ten-fold (Fig. 2). In addition, these CTL responses were very short-lived (not shown).

Induction of CTLs alone versus induction of antibodies alone

In a last series of experiments, we tried to induce either CTLs only and no antibodies specific for VSV-N, or vice versa, only antibodies and no CTLs. To avoid the induction of antibodies specific for the native VSV-N, we completely denatured recombinant VSV-N before immunization by boiling for 30 min. Denatured protein (50 μg) was sonicated and injected i.v. Although an efficient CTL response was induced (though reduced compared to native recombinant VSV-N), no antibodies specific for native VSV-N were detectable (Fig. 3). In contrast, if native recombinant VSV-N was injected, both CTLs and antibodies were generated (Fig. 3). Finally, if recombinant VSV-N was injected s.c. in incomplete Freund's adjuvant (IFA), a 30-fold reduced CTL response was generated, in contrast, to the sizable anti-VSV-N antibody response.

Discussion

It is generally assumed that exogenous antigens such as proteins or killed pathogens are taken up by phagocytic cells, degraded and presented on class II mole-

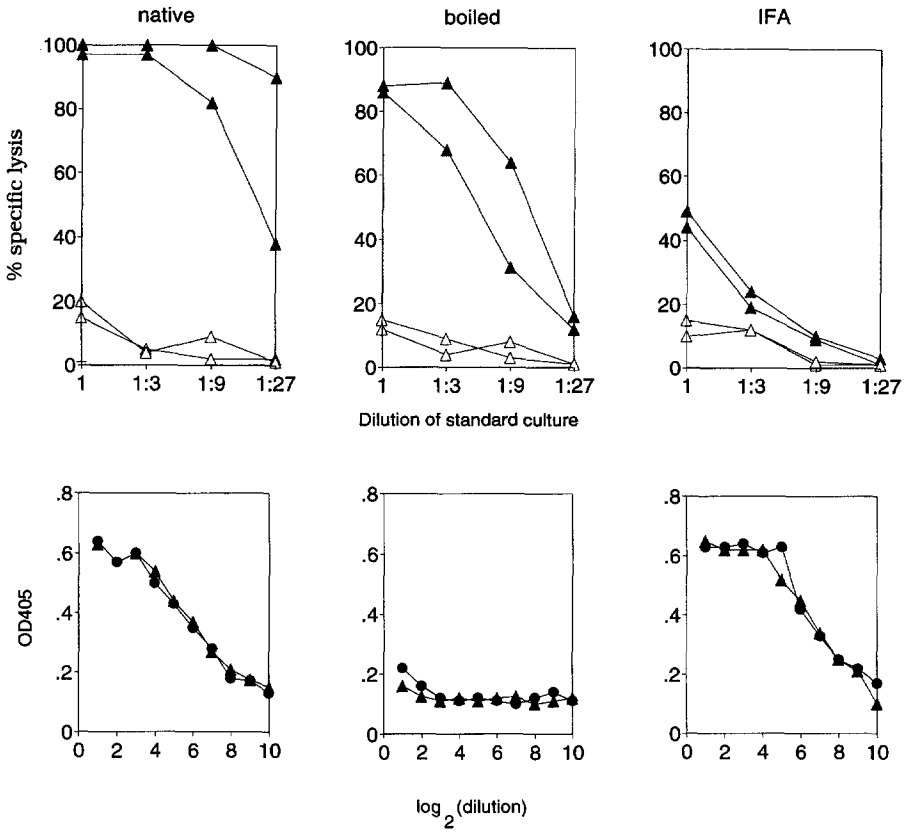

Fig. 3. Induction of specific CTLs alone or of specific antibodies alone. Mice were immunized with native VSV (10 μg), boiled recombinant VSV-N (50 μg) or native recombinant VSV-N (10 μg) in incomplete Freund's adjuvant. Twelve days later, spleens were removed and restimulated for 5 days with EL4 cells transfected to express VSV-N and tested in a standar ^{51}Cr-release assay on EL4 cells transfected to express VSV-N (*closed triangles*) or mock-transfected (*open triangles*) (Puddington et al. 1986) (*upper panels*). The same mice were bled on day 12 and the sera were tested for VSV-N-specific binding antibodies by ELISA (*lower row*). *Each line* represents an individual mouse. One of two similar experiments is shown

cules, leading to efficient induction of CD4$^+$ T helper cells. In contrast, intracellularly produced endogenous antigens reach the class I pathway and induce CTLs (Zinkernagel et al. 1977; Morrison et al. 1986; Germaine 1986). Recently, it has become evident that, although these are the major pathways, the class I and class II pathways are not absolutely separated; in some cases, particularly if relatively great amounts of antigen were used, endogenous proteins may reach the class II pathway (Germain and Margulies 1993) and exogenous proteins may reach the class I pathway (Rock et al. 1990; Staerz et al. 1987; Bevan 1987; Schirmbeck et al. 1992, 1994b; Bachmann et al. 1994b). The latter finding has important consequences for vaccine strategies, since live vaccines, leading to intracellular production of antigens, charging of class I molecules and induction of CTLs, may be dangerous in immunocompromised individuals (Horstmann 1979; Salk and Salk 1977). Because viruses are complex structures and protective antibodies and CTLs may

recognize epitopes on different proteins, we investigated whether vaccines can be generated by mixing the relevant target proteins for neutralizing antibodies and CTLs. Using the N of VSV (comprising the CTL epitope) and the G of VSV (exhibiting the neutralizing epitope) we found that such mixed vaccines indeed induced immune responses as efficiently as live virus itself.

To optimize immunization protocols, we tried different routes of immunization. Surprisingly, i.v. immunization was by far the most efficient route. It should, however, be taken into account that the recombinant protein used for immunization is injected together with cellular debris of the insect cell preparation used for production of the recombinant proteins. Such cellular debris have been shown to act as potent adjuvant for the generation of CTLs (Bachmann et al. 1994b, Bevan 1987). Most probably, the debris lead to a rapid sequestration of antigen in the spleen and phagocytosis of the proteins. Using ovalbumin coupled to beads, phagocytosis has been shown to be a key event for CTL priming (Kovacsovics-Bankowski et al. 1993); by analogy, it is probable that cellular debris function similarly.

Efficient vaccines induce both CTLs and antibodies. Nevertheless, under some circumstances, one or the other may not be desired, since antiviral antibodies or CTLs might cross-react with host proteins and cause immunopathology (Ada 1991; Oldstone et al. 1986). We found that denaturing of the protein used for priming prevents formation of significant amounts of antibodies recognizing the native protein, whereas CTLs were primed efficiently. We do not imply that denatured protein does not induce antibodies but rather that the antibodies induced cross-react poorly with the native form. In contrast, protein in IFA apparently induced only a poor CTL response but, as expected, mounted a good antibody response. This corroborates recent studies reporting that hepatitis S antigen in alum adjuvants induced antibodies but no CTLs (Schirmbeck et al. 1994a), whereas denatured antigen induced CTLs but virtually no specific antibodies (Schirmbeck et al. 1994b).

In conclusion, combinations of recombinant viral proteins may be used as vaccines to induces effector class-selected and -restricted immune responses.

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