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Formalin Inactivation of Vesicular Stomatitis Virus Impairs T-Cell- but Not T-Help-Independent B-Cell Responses

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The effects of formalin on the infectivity and immunogenicity of vesicular stomatitis virus (VSV) serotype Indiana were investigated. We found that formalin inactivation of VSV prevents infection of Vero cells in a concentration- and time-dependent manner, as shown by fluorometric cell analysis and inhibition of plaque formation. Inactivated VSV failed to induce significant cytotoxic T-lymphocyte responses in vivo or after restimulation in vitro. In contrast, the early immunoglobulin M (IgM) response, which is T help independent in the VSV system, was unaltered, suggesting normal antigenicity for and induction of B cells. However, no switch to IgG occurred, demonstrating failure of induction of T help. If cross-reactive T help was provided by previous infection with a second serotype of VSV (New Jersey), the IgG response was almost completely restored, confirming that the absence of IgG was due to lack of T help. A formalin-treated preparation of glycoprotein of VSV led to a delayed but otherwise normal IgG response, whereas treatment of VSV with UV light or β-propiolactone reduced IgG titers to the same extent as did formalin. These results suggest that loss of infectivity and the ensuing lack of amplification of viral antigens of formaldehyde-inactivated VSV is the major factor impairing induction of specific T-helper cell responses.

To be acceptable, vaccines must be safe to use; for this purpose, several standard procedures for vaccine preparation have been developed. Formalin inactivation of the pathogen has been used to inactivate poliovirus (9, 10), influenza virus (19), rabies virus (24), and more recently simian immunodeficiency virus (15). The major disadvantage of these vaccines is that their immunogenicity is reduced by the formalin treatment, leading to low antibody titers with an often restricted isotype pattern (9, 10). A second procedure to obtain safe vaccines is the preparation of attenuated agents which still replicate to a certain extent in the host. These vaccines usually lead to excellent, often life-long immunity and for this reason have often been preferred to inactivated vaccines, e.g., in the case of poliovirus (29). However, a disadvantage of live vaccines is that they may be safe in healthy individuals but cause severe complications in immunosuppressed individuals (9, 22, 30), such as AIDS patients. In addition, in some instances, live vaccines may regain their virulence (5, 22). Because of the high temperatures in tropical countries, the proper storage of live vaccines causes major problems, making large-scale vaccinations difficult and sometimes inefficient (9, 10). To better understand immunological consequences of formalin inactivation, we attempted to characterize the immune response of mice against formalin-inactivated vesicular stomatitis virus (VSV) serotype Indiana (VSV-IND) as a model system.

VSV belongs to the family *Rhabdoviridae* and is a close relative of rabies virus (34). In mice, VSV induces both a cytoxic T-lymphocyte (CTL) (28) and a neutralizing antibody response (11); the neutralizing immunoglobulin G (IgG) and not the CTL response seems to be crucial for recovery from primary infections (8, 16). The antibody response is characterized by an early T-help-independent IgM response peaking around day 4 (4), which is followed by a strictly T-help-dependent IgG response by days 6 to 8 (16).

We found that formalin-inactivated VSV induced a normal IgM response but that the T-cell responses responsible for the immunoglobulin class switch to IgG and the CTL response were drastically reduced or absent.

MATERIALS AND METHODS

Mice. Inbred C57BL/6 ($H\text{-}2^b$), B10.BR ($H\text{-}2^k$), BALB/c ($H\text{-}2^d$), ICR (+/+) ($H\text{-}2^q$), and ICR (*nu/nu*) ($H\text{-}2^q$) mice were obtained from the breeding colony of the Institut für Zuchthygiene, Tierspital Zürich, Zürich, Switzerland. Mice were between 8 and 12 weeks of age.

Viruses. VSV-IND (Mudd-Summer isolate) and VSV New Jersey (VSV-NJ) (Pringle isolate) seeds, originally obtained from D. Kolakofsky, University of Geneva, were grown on BHK-21 cells infected with a low multiplicity of infection and plaqued on Vero cells (18). The generation of recombinant vaccinia viruses expressing the nucleoprotein or glycoprotein of VSV-IND (vacc-IND-N and vacc-IND-G) has been described elsewhere (17). Vacc-IND-N and vacc-IND-G were gifts of B. Moss, Laboratory of Viral Diseases, National Institutes of Health, Bethesda, Md. Recombinant viruses were grown on BSC 40 cells at a low multiplicity of infection and plaqued on the same cells. The recombinant baculovirus expressing the glycoprotein of VSV-IND was a generous gift of D. H. L. Bishop, NERC Institute of Virology, Oxford, United Kingdom. It was derived from nuclear polyhedrosis virus and was grown at 28°C in *Spodoptera frugiperda* cells in spinner cultures in TC-100 medium.

Inactivation of VSV. Formalin inactivation of VSV-IND was performed for all in vivo experiments at 4°C for 18 h at a formalin (Merck, Darmstadt, Germany) concentration of 0.0625% in minimal essential medium supplemented with 1% fetal calf serum or, for in vitro experiments, as stated for the individual experiments. For inactivation, we used only high-

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titer virus preparations (10^9 PFU/ml) which could be diluted to reduce formalin to nontoxic concentrations after the inactivation procedure.

β-Propionolactone (Sigma) inactivation was performed under conditions previously determined to be optimal for complete inhibition of plaque formation at a concentration of 0.1% for 18 h at 4°C in minimal essential medium supplemented with 2% fetal calf serum.

A small volume of high-titer virus preparation was UV inactivated as a thin layer in a petri dish for 2 min under a UV lamp.

Serum neutralization test. The sera were prediluted 40-fold in supplemented minimal essential medium and 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. Then 100 μl of the serum-virus mixture 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 of Dulbecco modified Eagle medium containing 1% methylcellulose. After incubation for 24 h at 37°C, the overlay was flicked off and the monolayer was fixed and stained with 0.5% crystal violet. The highest dilution of serum that reduced the number of plaques by 50% was taken as the titer. Because of 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 (32). Unreduced samples were taken as IgM titers only if the corresponding reduced samples had at least a fourfold lower titer, i.e., when the IgG present in the unreduced sample could be neglected.

Detection of CTLs in vivo or in vitro. For in vitro detection of CTLs, C57BL/6 mice were infected with VSV-IND and spleens were removed 12 days later. Then 3×10^6 spleen cells from infected mice were cultivated for 5 days in the presence of 2×10^6 gamma-irradiated (3,000 rads) spleen cells, infected with UV-inactivated VSV (multiplicity of infection of 15) (28), or labeled with a peptide encompassing amino acids 49 to 62 (peptide 49-62) of the nucleoprotein of VSV (33). Cells were harvested and tested in a conventional 5-h ^{51}Cr release assay on EL-4 cells transfected with the nucleoprotein of VSV or with a control plasmid (25). For in vivo detection of CTLs, BALB/c (*H-2*^{d}) mice were infected with VSV-IND and challenged 6 to 14 days later with 5×10^6 PFU of vacc-IND-N or 5×10^6 PFU of vacc-IND-G intraperitoneally. Vaccinia virus titers in ovaries were determined 5 days later as described previously (2). Titers are shown as PFU per ovary.

Infection of cells. Vero cells were infected in suspension with VSV at a multiplicity of infection of 10 for 3 h at 37°C on a shaker. For fluorescence-activated cell sorting (FACS) analysis, cells were stained first with a mouse monoclonal antibody directed against the glycoprotein of VSV-IND (clone VI 22; unpublished data) and then with a fluorescein isothiocyanate-labeled goat anti-mouse antibody (TAGO, Burlingham, Calif.).

CD4 depletion of mice. Three days and 1 day before immunization with VSV, mice were given intraperitoneally two doses of 1 mg of YTS 191.1 (16) (the hybridoma cell line was a generous gift of H. Waldmann). The depleted $CD4^+$ cell population was below the detection level by FACS analysis. Functional depletion was confirmed by complete abrogation of the IgM-to-IgG switch of neutralizing antibodies against VSV (16).

Production of VSV-IND glycoprotein. To produce VSV-IND glycoprotein, *S. frugiperda* SF9 cells at a density of 2×10^6 cells per ml in spinner flasks were infected with recombinant baculovirus expressing the VSV-IND glycoprotein with a multiplicity of infection of 10 for 24 h at 28°C. Infected cells were harvested, disrupted by sonication, and stored at −20°C. The presence of glycoprotein was confirmed by Western immunoblot analysis, and the concentration of VSV-IND glycoprotein was estimated by sodium dodecyl sulfate-gel analysis.

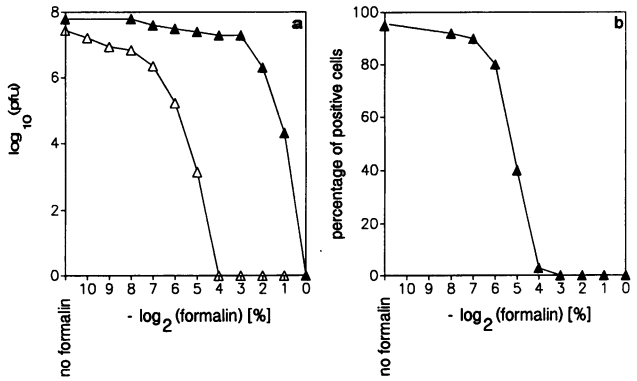

FIG. 1. Effects of formalin inactivation on replication (a) and infection (b) of cells by VSV-IND. (a) VSV-IND was incubated with different concentrations of formalin for 30 min (closed triangles) or 18 h (open triangles) at 4°C, and the number of remaining PFU was evaluated by plaquing on Vero cells. (b) VSV-IND was inactivated for 18 h at 4°C with different concentrations of formalin, and infectivity was determined by infection of Vero cells for 3 h at 37°C and subsequent FACS analysis of these cells stained with a VSV-IND glycoprotein-specific antibody. Infectivity is expressed as percentage of positive cells.

RESULTS

Formalin inactivation prevents VSV infection in vitro. VSV (10^9 PFU) was incubated for either 30 min or 18 h with different dilutions of formalin. The number of PFU present in the solution after inactivation was measured on Vero cell monolayers (Fig. 1a). As expected, the number of plaques decreased with higher formalin concentrations. If VSV was inactivated for 18 h, 0.0625% formalin reduced viral replication below detection levels, whereas after 30 min of inactivation, a concentration of 1% was needed for complete inhibition of plaque formation.

To determine whether formalin inactivation also prevented abortive infection of cells by VSV, Vero cells were incubated with VSV that had been inactivated for 18 h at 4°C with serial dilutions of formalin. After 3 h, infection of cells was determined by immunostaining for newly synthesized glycoprotein of VSV-IND expressed on the cell surface. As shown in Fig. 1b, glycoprotein expression was reduced by increasing formalin concentrations and was almost completely absent at a formalin concentration of 0.0625%, the same concentration that prevented plaque formation completely.

Induction of CTLs. To test the induction of in vitro restimulatable CTLs, C57BL/6 (*H-2*^{b}) mice were infected with 2×10^4 PFU of live or 2×10^6 or 10^8 PFU of inactivated VSV-IND; 12 days later, spleen cells were cultivated in the presence of 2×10^6 irradiated spleen cells infected with VSV or labeled with the relevant CTL peptide derived from the nucleocapsid of VSV (peptide 49-62) (33) for 5 days. The

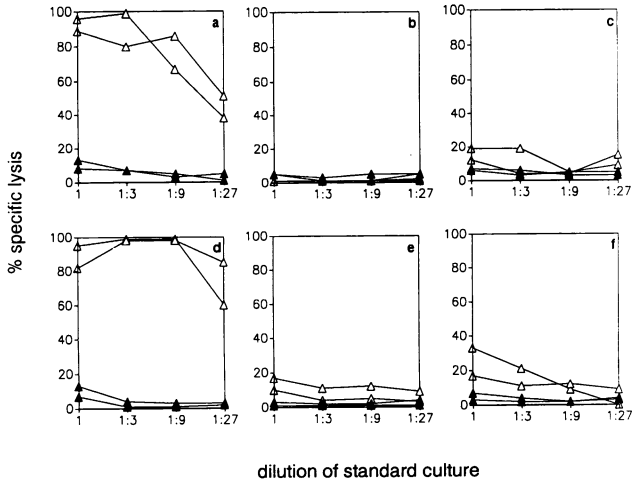

FIG. 2. Evidence that formalin treatment of VSV-IND abrogates induction of CTLs. C57BL/6 mice were immunized with 2×10^4 PFU of live (a and d) or 2×10^6 (b and e) or 10^8 PFU (c and f) of inactivated VSV-IND, and spleens were removed 12 days later. Cells were incubated then in the presence of 2×10^6 spleen cells infected with VSV (a to c) or labeled with peptide 49-62 (33) (d to f) and tested in a ^{51}Cr release assay on EL-4 cells transfected with the nucleoprotein of VSV (open triangles) or transfected with a control plasmid (25) (closed triangles). Results of two individual mice per group are shown.

presence of CTLs was assessed in a ^{51}Cr release assay (Fig. 2). Untreated VSV induced a strong CTL response (Fig. 2a and d), but formalin treatment of VSV-IND abrogated the induction of CTLs to barely detectable levels (Fig. 2b, c, e, and f). The same results were obtained for BALB/c ($H\text{-}2^d$) mice (not shown).

Later time points were not tested, since primary anti-VSV CTL activity is maximal 6 days after infection with virus (28) and the presence of restimulatable precursors of CTLs therefore can be expected to be maximal around that time and a few days later (21).

A second, very sensitive assay for CTL detection in vivo was used as an additional readout (2, 3). BALB/c mice were immunized with 5×10^4 PFU of live or 5×10^4 or 2×10^7 PFU of inactivated (18 h, 0.0625% formalin) VSV-IND and challenged intraperitoneally with 5×10^6 PFU of vacc-IND-N or vacc-IND-G on day 6 to 14. Vaccinia virus titers in ovaries were determined 5 days after the challenge infection (Table 1). Reduction of vaccinia virus titers in primed mice compared with unprimed control mice has been shown to be mediated by VSV-specific $CD8^+$ CTLs in $H\text{-}2^d$ mice (2, 12). Priming with live VSV-IND led to complete protection against vacc-IND-N and vacc-IND-G, whereas mice primed with inactivated VSV-IND had vaccinia virus titers in ovaries as high as those in unprimed control animals (Table 1). Formalin inactivation of VSV therefore abrogated the induction of biologically relevant CTLs against both the glycoprotein and nucleoprotein of VSV-IND.

Induction of B-cell responses. VSV is known to induce a T-help-independent IgM peaking around day 4 and then a strictly T-help-dependent IgG appearing between days 6 and 8 and peaking around day 21. To determine whether formalin inactivation of VSV had an influence on its B-cell immunogenicity, BALB/c mice were immunized with different doses of VSV-IND (2×10^4, 2×10^6, and 10^8 PFU), either live (Fig. 3a and c) or inactivated (Fig. 3b, d, and e); blood was

TABLE 1. Elimination of vacc-IND-N and vacc-IND-G from ovaries in mice primed with live or inactivated VSV-IND[a]

Mouse group	Vaccinia virus titer (mean $\log_{10}$ ± SD) in ovaries	
	Vacc-IND-N	Vacc-IND-G
Primed with:		
5×10^4 PFU of live VSV-IND	<0.7	<0.7
5×10^4 PFU of inactivated VSV-IND	5.8 ± 0.6	5.4 ± 0.3
2×10^7 PFU of inactivated VSV-IND	5.0 ± 1.75	6.0 ± 1.75
Unprimed control	5.3 ± 0.4	5.6 ± 0.5

[a] Mice were primed on day zero with 5×10^4 PFU of live or 5×10^4 or 2×10^7 PFU of formalin-inactivated VSV-IND and challenged 6 to 14 days later with 5×10^6 PFU of vacc-IND-N or vacc-IND-G. Ovaries were removed 5 days later and homogenized, and virus titers were determined in three mice per group.

taken on days 4, 8, 12, and 21 thereafter. Formalin inactivation had no influence on the early IgM response but drastically reduced the switch from IgM to IgG; 2×10^4 PFU of inactivated virus did not induce any measurable neutralizing IgG titer (Fig. 3b), compared with a titer of 1:40,000 in mice immunized with 2×10^4 PFU of live virus (Fig. 3a). At a higher dose, 2×10^6 PFU, inactivated VSV induced some neutralizing IgG (Fig. 3d) but about 200-fold less efficiently than did live virus (Fig. 3c); 10^8 PFU of inactivated virus, however, induced a strong antibody response with almost normal neutralizing IgG titers (Fig. 3e). This dose of VSV

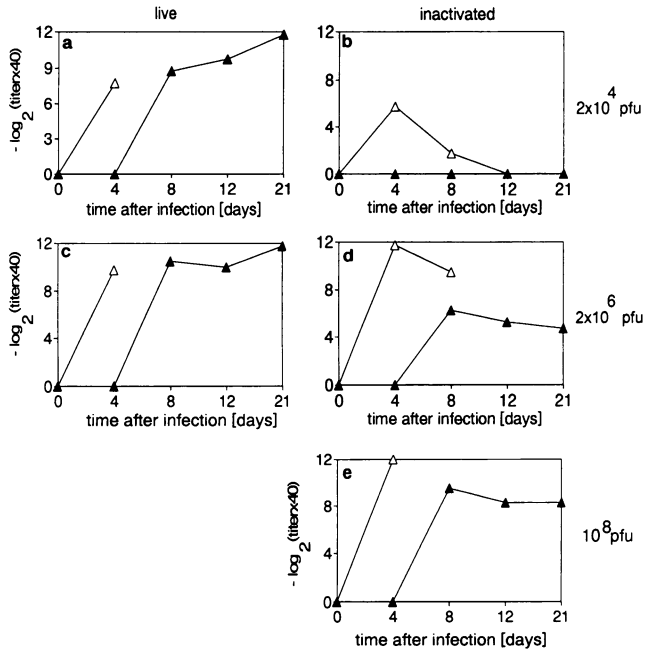

FIG. 3. Induction of B-cell responses by formalin-inactivated VSV-IND. Mice were immunized with 2×10^4 (a and b) or 2×10^6 (c and d) PFU of either live (a and c) or inactivated (b and d) VSV-IND. In panel e, mice were infected with 10^8 PFU of inactivated virus. Neutralizing IgM (open triangles) and IgG (closed triangles) antibody titers were determined in three mice per group on days 4, 8, 12, and 21. Means are shown; standard errors of the means were less than one dilution step.

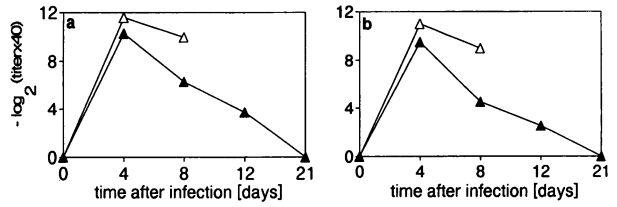

FIG. 4. Induction of neutralizing IgM by inactivated VSV-IND in the absence of T help. CD4-depleted (closed triangles) and normal (open triangles) BALB/c mice (a), ICR (*nu/nu*) (closed triangles), and ICR (+/+) (open triangles) mice (b) were immunized with 2×10^6 PFU of inactivated VSV-IND, and neutralizing IgM titers were determined from three mice per group on days 4, 8, 12, and 21. Means are shown; standard errors of the means were less than one dilution step.

was not tested with live virus, since mice usually do not survive such a high dose of VSV.

To examine whether formalin might render the IgM response of VSV T help dependent, either anti-CD4-treated mice (Fig. 4a) or nude mice (ICR *nu/nu*) (Fig. 4b) were injected intravenously with 2×10^6 PFU of formalin-inactivated VSV; neutralizing IgM antibody titers were determined on days 4, 8, 12, and 21. After formalin inactivation of VSV, the IgM in anti-CD4-depleted mice (Fig. 4a) or nude mice (Fig. 4b) was not impaired, confirming that formalin inactivation did not alter B-cell antigenicity and immunogenicity. As expected, no IgG was detected.

Restauration of the neutralizing IgG response by cross-reactive T help. The absence of an efficient switch from IgM to IgG suggested that formalin-inactivated VSV-IND cannot induce T help (16). We therefore tried to restore the antiviral IgG response, using VSV-NJ as a source of cross-reactive T help (26). VSV-NJ is known to induce an efficient B-cell response in mice comparably to VSV-IND, but neutralizing antibodies do not cross-react between the two serotypes (13, 14). However, it has been shown that T help induced by VSV-NJ can enhance anti-VSV-IND T-helper cell activity and therefore is cross-reactive (26); B10.BR mice were immunized on day −4 with 2×10^6 PFU of live VSV-NJ and on day 0 with 2×10^5 PFU of either live or inactivated VSV-IND. Mice preinfected with VSV-NJ developed high anti-VSV-IND IgG titers, irrespective of whether they were immunized with live (Fig. 5a) or inactivated (Fig. 5b) VSV-IND. In contrast, control animals, which had not been primed with VSV-NJ, showed high anti-VSV-IND IgG titers only when they were immunized with live VSV-IND.

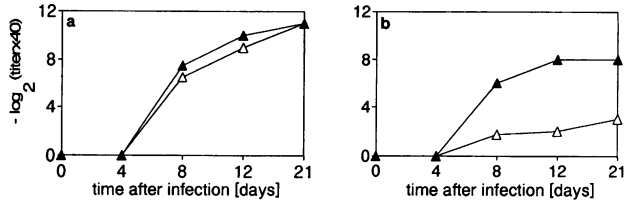

FIG. 5. Induction of B-cell response by formalin-inactivated VSV-IND in the presence of cross-reactive T help. B10.BR mice were infected on day −4 with 2×10^6 PFU of live VSV-NJ (closed triangles) or left untreated (open triangles) and immunized on day 0 with 2×10^5 PFU of live (a) or inactivated (b) VSV-IND; VSV-IND neutralizing IgG titers were determined in three mice per group on days 4, 8, 12, and 21 after immunization with VSV-IND. Means are shown; standard errors of the means were less than one dilution step.

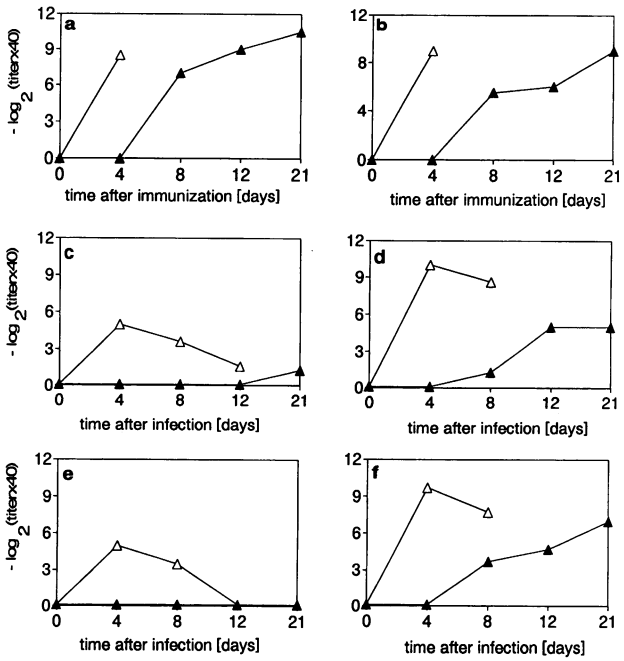

FIG. 6. Evidence that reduced IgG titers are due to loss of infectivity. Glycoprotein of VSV-IND was formalin treated as described for virus inactivation (0.0625% formalin for 18 h at 4°C). Mice were immunized with 10 μg of untreated (a) or formalin-treated (b) glycoprotein. VSV-IND was treated with β-propiolactone (c and d) or UV light (e and f), and mice were infected with a dose corresponding to 2×10^4 (c and e) or 2×10^6 (d and f) PFU before inactivation. Neutralizing IgM (open triangles) and IgG (closed triangles) antibody titers were determined in three mice per group on days 4, 8, 12, and 21. Means are shown; standard errors of the means were less than one dilution step.

Loss of T help is due to loss of infectivity. To determine whether it was the loss of viral replication or rather the alteration of T-helper cell epitopes by formalin that abrogated T help, a VSV-IND glycoprotein preparation was treated with formalin as described for VSV. BALB/c mice were immunized intravenously with 10 μg of either untreated or formalin-treated glycoprotein in balanced salt solution without adjuvant. Neutralizing antibody titers were measured on days 4, 8, 12, and 21 (Fig. 6). Formalin treatment of VSV glycoprotein only delayed the IgG response but did not reduce significantly neutralizing IgG titers on day 21 after immunization.

To rule out the possibility that the observed effects of formalin inactivation were due to inappropriate processing in the endosome by cross-linking of proteins or inhibition of the fusion of the virus with the cell membrane, thus reducing T help, two additional inactivation procedures were tested: treatment with β-propiolactone (Fig. 6c and d) and UV light (Fig. 6e and f). β-Propiolactone is assumed to interact only with RNA (6) and not with proteins; UV light is known to cross-link RNA but not proteins and does not appear to interfere with the fusion properties of the virus (23, 27). Both treatments affected the B-cell response to VSV in the same way as did formalin, ruling out a critical influence of protein cross-linking on the induction of T help (Fig. 6c to f).

DISCUSSION

This study characterized the effect of formalin inactivation on the immunogenicity of VSV. The minimal concentration for efficient inactivation was 0.0625% for 18 h at 4°C. Immunogenicity of inactivated VSV for CTL was evaluated in vitro by secondary restimulation of spleen cells or in vivo by a CTL-dependent protection assay against vaccinia virus-VSV recombinants (2). Formalin treatment of VSV completely abrogated CTL induction, as demonstrated by the absence of in vitro restimulatable CTLs and protection against vaccinia virus-VSV recombinants, probably because noninfectious viral antigens do not usually enter the class I pathway under physiological conditions (7, 20). The absence of CTL induction seems to be a major disadvantage of inactivated vaccines and can probably be circumvented only by vaccination with a recombinant vaccinia virus or by coimmunization with an appropriate class I CTL peptide (1).

In contrast to the loss of CTL induction, B-cell antigenicity and immunogenicity were not impaired by formalin inactivation; live and inactivated virus induced comparable IgM responses in normal, CD4-depleted, and nude mice. However and very importantly, formalin inactivation of VSV completely abrogated the immunoglobulin class switch from IgM to IgG if mice were immunized with low VSV doses (2×10^4 PFU); IgG was drastically reduced if mice were immunized with higher VSV doses (2×10^6 PFU). That the abrogation of the immunoglobulin class switch was due to the absence of T help was confirmed by successful reconstitution of the IgG responses by cross-reactive T help induced by prior infection with VSV-NJ. In fact, priming with VSV-NJ 4 days before immunization with inactivated VSV-IND restored the neutralizing anti-VSV-IND IgG response to almost normal levels. T help may possibly also be provided either by coinfection with a second harmless viral strain exhibiting the relevant determinants or by appropriate coimmunization with a T-helper cell peptide (31).

We have investigated several possible reasons for the lack of T help: (i) relevant T-helper epitopes could be chemically altered by formalin, (ii) cross-linking of proteins by formalin could prevent appropriate processing in the endosome or inhibit fusion activity of the glycoprotein, or (iii) infection is prevented and therefore the amount of antigen is reduced. The chemical alteration of T-helper epitopes was excluded as a relevant factor, since formalin-treated VSV-IND glycoprotein was able to induce normal IgG responses. The induction of cross-links by formalin could also be excluded as an important factor for the loss of T help, because UV light, which does not cross-link proteins and presumably does not inhibit membrane fusion activity (23, 27), reduced IgG titers as completely as does formalin treatment. These results were fully confirmed by the identical results with β-propiolactone-inactivated VSV, since, like UV light, β-propiolactone seems to act dominantly on RNA and not on proteins (6). For formalin-inactivated VSV, therefore, the key factor reducing the IgG response seems to be loss of infectivity. This correlation between loss of infectivity and lack of T help probably reflects the limiting presence of viral antigens, because inactivated VSV does not amplify viral antigens during an abortive replication cycle. Accordingly, immunization of mice with 10^8 PFU of inactivated virus, corresponding roughly to about the 1 μg of viral glycoprotein, led to IgG titers comparable to those obtained after immunization with VSV glycoprotein alone. This result corroborates findings for humans, in which case low antibody titers obtained after vaccination with inactivated poliovirus or influenza virus could be increased by greater amounts of antigen used for immunization (30).

In conclusion, formalin inactivation of VSV led to a selective loss of immunogenicity for CTLs and a drastic reduction of induction of T-helper cells but did not impair B-cell immunogenicity. Neutralizing IgG responses could be restored by additional induction of cross-reactive T help or by increased vaccine doses. These results indicate that a high dose of minimally formaldehyde-inactivated VSV represents a useful vaccine for induction of B-cell responses, but it fails to induce an efficient CTL response. This imbalance may be of disadvantage in viral infections, for which both antibodies and CTL responses are necessary for efficient protection.

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