Antibodies against the Two Serotypes of Vesicular Stomatitis Virus Measured by Enzyme-Linked Immunosorbent Assay: Immunodominance of Serotype-Specific Determinants and Induction of Asymmetrically Cross-Reactive Antibodies

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The serological relationship between the two vesicular stomatitis virus (VSV) strains Indiana (VSV-Ind) and New Jersey (VSV-NJ) were analyzed by using an enzyme-linked immunosorbent assay (ELISA). Immunoglobulin G responses, defined by their resistance to treatment with 2-mercaptoethanol, were assessed by ELISA by using sucrose gradient-purified VSV or purified VSV glycoproteins (G) as antigens. When low doses (10^6 PFU) of live VSV or 10^8 PFU of UV-inactivated virus were given intraperitoneally (i.p.), only non-cross-reactive antibody responses were observed in a primary immune response. However, when 10^6 PFU of live VSV were injected intravenously (i.v.), cross-reactive antibodies were generated; anti-VSV-NJ antibodies cross-reacted more against VSV-Ind than did anti-VSV-Ind antibodies against VSV-NJ. When 10^8 PFU of live VSV or UV-inactivated VSV mixed with complete Freund adjuvant was given i.p., high levels of cross-reactive antibodies detectable by ELISA were induced in primary and secondary responses. When purified G protein was used instead of purified whole virus in the ELISA, the cross-reactivity was found to be asymmetrical after immunization with live VSV given i.v. but not after i.p. inoculation; anti-VSV-NJ sera bound almost equally well to VSV-Ind G protein, whereas anti-VSV-Ind sera bound virtually exclusively to the G protein of the homologous serotype. The data suggest that immunization with VSV given i.p. results in a more specific, i.e., less cross-reactive, response than that either after i.v. infection or after the virus antigen is made available in great amounts or if it persists for prolonged periods when given i.p. together with complete Freund adjuvant. The unique determinants were immunodominant because they induced antibodies preferentially, whereas partially shared determinants induced antibody responses asymmetrically, more slowly, and with lower titers. Interestingly, the asymmetric cross-reactivity of anti-VSV antibodies, as measured by ELISA, against purified VSV G was opposite that observed for cytotoxic T cells.

Vesicular stomatitis virus (VSV) is a rhabdovirus with a bullet-shaped structure which is covered with surface projections of glycoproteins (Gs). The determinants binding antibodies that can neutralize the virus was located on these Gs (6). With respect to these neutralizing antibodies, VSV has been classified into two non-cross-reactive serotypes, VSV-Indiana (VSV-Ind) and VSV-New Jersey (VSV-NJ) (6). Nevertheless, some determinants are shared between the Gs of the two serotypes and can be detected by the enzyme-linked immunosorbent assay (ELISA) (2, 7, 8). Thus, the G of VSV-Ind and VSV-NJ serotypes contain neutralizing determinants that are unique and of direct relevance in protection; others do not contribute to protective immunity but induce cross-reactive antibodies which are detectable in an ELISA. Several studies on VSV-specific cytotoxic T cells have shown that, in contrast to neutralizing antibodies, T cells are cross-reactive. Primary VSV-Ind-immune T cells cross-react more extensively against VSV-NJ-infected cells than do VSV-NJ-immune T cells against VSV-Ind-infected target cells, whereas secondary cytotoxic T cells cross-react more or less symmetrically (10, 11, 14-16).

In this study we analyzed by ELISA whether antibodies generated during immunization with VSV correlated with

the cross-reactivities seen with cytotoxic T cells. The results of these studies show that intraperitoneal (i.p.) immunization with UV-inactivated virus (10^8 PFU) and live virus (10^6 PFU) induces mainly serotype-specific positive by ELISA or neutralizing antibodies; this indicates that neutralizing determinants are immunodominant. To elicit antibody responses against the shared and nonneutralizing determinants, mice had to be immunized intravenously (i.v.) with 10^6 PFU of live virus or i.p. with 10^8 PFU of live virus. Furthermore, we observed that the cross-reactivity of antibodies, as assessed by ELISA, on purified VSV was more or less symmetrical but was asymmetrical when assayed on purified G; anti-VSV-NJ antibodies bound to homologous and heterologous VSV G, whereas anti-VSV-Ind antibodies bound virtually exclusively to homologous VSV G. This asymmetry was opposite that described for primary cytotoxic T cells (10, 15).

MATERIALS AND METHODS

Mice. DBA/2 mice were obtained from the Institut für Zuchtgenetik, Zurich; mice were of either sex and were 8 to 10 weeks old when used for the experiments.

Virus. VSV-Ind (Mudd-Summer isolate) and VSV-NJ (Pringle isolate) virus stocks were prepared in BHK-21 cells by infection at a low multiplicity of infection, as described in detail elsewhere (1). Virus was concentrated by ultracentrifugation and purified on a sucrose density gradient by established methods (3, 6).

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FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified VSV and VSV G preparation used for immunization. Lanes 1 and 10, proteins used as molecular weight markers, from top to bottom: phosphorylase *b*, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; α -lactalbumin, 20,000; lanes 2 and 3, 4×10^9 and 1×10^9 PFU of purified VSV-Ind, respectively; lanes 4 and 5, 40 and 10 μ g of purified VSV-Ind G, respectively; lanes 6 and 7, 4×10^9 and 1×10^9 PFU of purified VSV-NJ, respectively; lanes 8 and 9, 40 and 10 μ g of purified VSV-NJ G, respectively. kD, Kilodaltons.

Preparation of purified surface G. The method used for preparing purified G has been described by Kelly et al. (6). Briefly, sucrose gradient-purified VSV-Ind and VSV-NJ was treated with Triton X-100 at a concentration of 2%. After treatment for 60 min at room temperature with constant stirring, the Triton X-100-virus mixture was centrifuged at $140,000 \times g$ for 90 min at 5°C . The supernatant containing G was analyzed by electrophoresis on a 12.5% sodium dodecyl sulfate-polyacrylamide gel (3, 9) by using Coomassie blue as a stain. When 10 to 50 μ g was analyzed no bands other than that for G could be detected (Fig. 1).

Immunization. A total of 10^6 or 10^8 PFU of live or UV-inactivated virus or purified G equivalent to the amount contained in 10^8 PFU with or without complete Freund adjuvant (CFA) was injected i.p. or i.v., as stated specifically for each experiment. To enhance antibody responses against the cross-reactive common determinants on the two VSV serotypes and on the respective Gs, mice preimmunized with whole virus or purified G of homologous and heterologous serotypes were challenged with purified Gs.

ELISA. The ELISA procedure adopted for this study was a modification of the assay described previously (1, 13).

Accordingly, 0.5 μ g of purified VSV per 100 μ l of carbonate-bicarbonate buffer (pH 9.6) was adsorbed per well of a 96-well polystyrene microtiter plate (type F; Petraplastic, Inotech, Wohlen, Switzerland) at 4°C overnight. The plates were washed five times with phosphate-buffered saline (pH 7.2) containing 0.05% Tween-20, and then 100 μ l of serial dilutions of sera in phosphate-buffered saline-0.05% Tween 20 was added to each well and incubated for 2 h at room temperature. Plates were washed again, and 100 μ l of goat anti-mouse immunoglobulin G (IgG) labeled with horseradish peroxidase (Tago 4143; Burlingame, Calif.) diluted optimally in phosphate-buffered saline-0.05% Tween 20 was added and then incubated for 2 h at room temperature. The plates were washed again, and 200 μ l of substrate containing 2 mg of 2,2'-azino-di-3-ethyl benzthiazoline sulfonate (Boehringer GmbH, Mannheim, Federal Republic of Germany) (1) in 20 ml of 0.1 M NaH_2PO_4 (pH 4.0) with 15 μ l of 30% H_2O_2 was added to each well. After 30 min of incuba-

FIG. 2. Binding of 2-mercaptoethanol-resistant antibodies to purified VSV-Ind measured by ELISA in sera from mice infected i.v. with 10^6 PFU of live VSV-Ind (■) or VSV-NJ (▨) during a primary (A) and a secondary (B) infection. Pooled serum samples from three to five mice were reacted on micro-ELISA plates coated with purified VSV-Ind.

tion at room temperature, all samples and appropriate controls were measured at 405 nm with a micro-ELISA reader (Virion AG, Rüsschlikon, Switzerland).

For the determination of anti-VSV-Ind G or anti-VSV-NJ G antibody titers by ELISA, the plates were coated with the optimal dilution (predetermined by checkerboard titrations and corresponding to about 1 to 3 μ g per well) of purified G; otherwise, the procedure was the same as described above.

RESULTS

Primary and secondary antibody response to 10^6 PFU of live VSV-Ind or VSV-NJ: cross-reactive antibodies against purified VSV detected by ELISA after i.v. but not after i.p. immunization. When DBA/2 mice were infected with 10^6 PFU of live VSV-Ind or VSV-NJ, they generated anti-VSV IgG antibodies that bound preferentially to purified VSV of the homologous strain but also to a significant extent to the heterologous VSV (Fig. 2A and 3A). For mice infected with VSV-Ind the titers on homologous virus were five steps of twofold dilutions greater than that on heterologous virus; for mice infected with VSV-NJ the difference was two to four steps, reflecting an even greater cross-reactivity. After secondary injection with homologous VSV-Ind, immune mice had titers of 1:20,480 to 1:41,960 on homologous VSV-NJ and up to 1:5,120 on heterologous VSV-NJ (Fig. 2B), i.e., a

FIG. 3. The same serum samples shown in Fig. 2 were tested by ELISA on purified VSV-NJ.

preference of three to five steps of twofold dilutions. For VSV-NJ, titers in immune mice reached 1:81,920 on homologous VSV-NJ and about 1:1,280 on heterologous VSV-Ind (Fig. 3B); the difference also represented about five to six titration steps.

When normal mice were infected with the same dose of 10^6 PFU of live VSV-Ind given i.p., antibody titers reached 1:2,560 on purified homologous virus but $<1:80$ on heterologous VSV (Fig. 4A and 5A). Mice infected with 10^8 PFU of live VSV-Ind, however, developed higher anti-VSV-Ind titers of 1:10,240 (day 13) to 1:20,480 (day 35), whereas titers on heterologous VSV-NJ reached 1:160 (day 13) and 1:640 (day 35) (Fig. 4B and 5B). Mice infected i.p. with 10^6 PFU of live VSV-NJ showed comparable titers on homologous purified VSV; they also lacked cross-reactive antibodies (Fig. 4A and 5A). Mice infected i.p. with 10^8 PFU of live VSV-NJ developed titers of 1:5,120 (day 13) to 1:20,480 (day 35) against VSV-NJ, with cross-reactive titers of 1:80 on VSV-Ind (day 13) rising to 1:640 by day 35 (Fig. 4B). With both viruses, antibody preferentially bound to purified homologous VSV on the order of or greater than five to six dilution steps higher after infection with 10^6 PFU and six dilution steps higher after infection with 10^8 PFU of live virus given i.p.

Induction of cross-reactive anti-VSV antibodies detected by ELISA after i.p. immunization with purified VSV plus CFA or with purified VSV G plus CFA. DBA/2 mice immunized i.p. with UV-inactivated VSV-Ind (equivalent to 10^8 PFU) mixed with CFA and boosted with the same amount of UV-inactivated VSV-Ind administered i.v. generated high titers of IgG that bound to the same extent to VSV-Ind- and VSV-NJ-coated plates (Table 1). Mice immunized and boosted identically with UV-inactivated VSV-NJ generated antibodies that bound to purified VSV-NJ to a titer of about 1:80,000 and to VSV-Ind to a titer of about 1:10,000; this revealed a significant level of cross-reactivity of the antibodies that were generated. Mice primed with purified VSV G in CFA and boosted with homologous VSV G showed antibody titers of 1:80,000 when tested against homologous VSV G; the cross-reactive antibody titers against heterologous VSV G were three to four dilution steps lower (Table 1).

Asymmetry of cross-reactivity of antibodies induced by live VSV given i.v. when assessed by ELISA by using purified VSV-G. Serum samples from DBA/2 mice infected with 10^6 PFU of live VSV-Ind or VSV-NJ were tested by ELISA on

FIG. 4. ELISA on purified VSV-Ind of serum samples from mice infected i.p. with 10^6 PFU of live VSV-Ind (■) or live VSV-NJ (▨) (A) and 10^8 PFU of live VSV-Ind (■) or live VSV-NJ (▨) (B). 2-Mercaptoethanol-resistant anti-VSV-Ind titers in pooled serum samples from three to five mice were monitored for up to 35 days after primary injection.

FIG. 5. The serum samples for which the assay results are shown in Fig. 4 were tested by ELISA on purified VSV-NJ.

purified VSV G of VSV-Ind or VSV-NJ origin (Fig. 6). Antibodies from VSV-Ind-primed mice bound strongly to VSV-Ind G but not to VSV-NJ G, whereas VSV-NJ-immune serum bound as strongly to VSV-Ind G as to VSV-NJ G. Mice immunized twice with purified VSV-Ind G or VSV-NJ G generated antibodies that bound exclusively to the respective purified Gs (Fig. 7).

DISCUSSION

The results of this study are summarized in Table 2. They show that mice immunized i.v. with 10^6 PFU of VSV of either serotype during primary or secondary responses generated antibodies which reacted with purified whole virus of the homologous serotype and also to a significant extent with VSV of the heterologous serotype. More or less symmetrical cross-reactivities were induced by 10^8 PFU of live VSV injected i.p.; high levels of cross-reactivity were caused by 10^8 UV-inactivated VSV in CFA given i.p. These findings are in contrast with the absence of significant levels of cross-reactivity in sera of mice immunized i.p. with 10^6 PFU of live VSV. Overall, specific, i.e., non-cross-reactive, responses were obtained readily by all the various injection protocols, particularly with low doses of VSV, suggesting that either the biologically most important, i.e., neutralizing (2, 12), determinants or possibly other unique determinants, or both, are immunodominant. Epitopes that are either shared naturally or that are possibly generated during purification procedures by partial denaturation may account for the cross-reactivity; these latter kinds of epitopes are able to induce antibody responses only if live virus is injected i.v., if live virus is given i.p. in amounts 100 times greater than those of live virus injected i.v., or if UV-inactivated VSV is given i.p. together with CFA. Moreover, the responses against the shared determinants usually appeared somewhat slower, and the end titers were lower than those of the non-cross-reacting binding antibodies. In general, the data suggest that high doses of virus or the long persistence of viral antigens due to CFA, or both, favor induction of cross-reactive antibodies.

The differences in the generation of cross-reactive antibodies with regard to routes of immunization (i.v. and i.p.) with 10^6 PFU of live virus could be due to differences in the extent of replication of VSV or in the level of antigen expression by antigen-presenting cells, or both. Also, the postulated direct mitogenic action of VSV on murine B cells may be responsible for the reason that mice immunized i.v. but not i.p. generated cross-reactive antibody responses; the more direct exposure to and subsequent proliferative re-

TABLE 1. Both UV-inactivated VSV and purified VSV G mixed with CFA induce cross-reactive antibody responses measured by ELISA by using whole VSV as substrate

Primary injection ^a	Secondary injection	Titer by ELISA on purified whole ^b :	
		VSV-Ind	VSV-NJ
10 ⁸ PFU of VSV-Ind (UV-inactivated) and CFA	10 ⁸ PFU of VSV-Ind (UV-inactivated)	1/81,920	1/81,920
10 ⁸ PFU of VSV-NJ (UV-inactivated) and CFA	10 ⁸ PFU of VSV-NJ (UV-inactivated)	1/10,240	1/81,920
None	10 ⁸ PFU of VSV-Ind (UV-inactivated)	1/640	1/80
None	10 ⁸ PFU of VSV-NJ (UV-inactivated)	1/80	1,640
20 µg of VSV-Ind G and CFA	20 µg of VSV-Ind G	1/81,920	1/5,120
20 µg of VSV-NJ G and CFA	20 µg of VSV-NJ G	1/10,240	1/81,920

^a Mice were primed i.p. as indicated; 6 weeks later they were challenged i.v. and bled on day 8.

^b Values are for pooled serum samples from three to five mice.

response of B cells after i.v. immunization with VSV may cause a more heterogeneous antibody response than when the same amount of virus is given i.p. (5).

Whereas the antibodies induced by live VSV cross-reacted symmetrically in an ELISA with whole virus, the same antisera assayed by ELISA on purified G revealed an asymmetrical pattern of cross-reactivity: Anti-VSV-NJ sera bound to VSV-Ind G antigen, whereas anti-VSV-Ind sera did not significantly bind to VSV-NJ G antigen. This pattern was found to be independent of the form of antigen used for priming: live VSV, UV-inactivated VSV, or VSV G with CFA. If VSV G without CFA was used to prime mice, however, a secondary response to VSV G lacked cross-reactive antibodies, even in mice primed and challenged with VSV-NJ G (Fig. 7). This asymmetry was not caused by contamination with common antigens in the purified VSV G preparation, particularly of the G of the VSV-Ind serotype, for the following reasons. First, the G preparations were monitored at various concentrations by sodium dodecyl sulfate-gel electrophoresis and at up to 40 µg per run showed no band smaller than the G band (Fig. 1). Second, hyperimmunization (in CFA) with VSV-Ind G preparations revealed no greater cross-reactivity on purified VSV-NJ or VSV-NJ G than did sera from mice hyperimmunized with VSV-NJ G on purified VSV-Ind or VSV-Ind G (Table 1). If the VSV-Ind G preparations that were used were contaminated more with shared constant antigenic determinants on viral antigens other than G, the results reported in Table 1 would be difficult to explain. Hyperimmune anti-VSV-Ind G antibod-

ies bound to purified VSV-NJ at titers approximately similar to those as anti-VSV-NJ G antisera that were cross-reacted on VSV-Ind, while both antisera reached comparable titers by ELISA on the purified homologous VSV. Had the contamination of VSV-Ind G been more extensive, then a higher cross-reactive titer on purified VSV-NJ than that reached by the antiserum against the purer VSV-NJ G preparation would have been expected. Because this was not the case, it must be argued that common antigens used in ELISA are less accessible on purified VSV-NJ than on purified VSV-Ind. Although this speculation cannot be excluded formally, the following findings do not readily support it. First, cross-reactive antibodies induced by UV-inactivated VSV-Ind plus CFA i.p. bound better to purified VSV-NJ (Table 1) than did antibodies against UV-inactivated VSV-NJ plus CFA on purified VSV-Ind; second, and more indirectly, purified and inactivated VSV-NJ with or without CFA induced cross-reactive antibodies that bound to VSV-Ind G to much higher titers than did antibodies to purified VSV-Ind. Third, hyperimmune serum against the G preparation neutralized only homologous virus (data not shown).

When assessed by ELISA, the difference in cross-

FIG. 6. Serum samples from mice infected i.v. with 10⁶ PFU of live VSV-Ind (■) or VSV-NJ (▨) were assayed by ELISA on purified VSV G for anti-VSV-Ind G (A) and anti-VSV-NJ G (B) antibodies.

FIG. 7. Patterns of serological cross-reactivity assessed by ELISA on purified VSV G. Two pools of serum samples from two to four mice immunized i.v. with VSV-Ind G (■) or VSV-NJ G (▨) twice at an interval of 3 weeks were assayed on purified VSV-Ind G and VSV-NJ G.

TABLE 2. Summary of the data obtained

2-Mercaptoethanol-resistant antibodies measured by ^a :													
Primary immunization		Secondary immunization		Neutralization assay for:		ELISA				Cytotoxic T-cell ^b activity against target cells infected with:			
VSV strain (dose [PFU])	Type of virus or treatment (route of injection)	VSV strain (dose [PFU])	Type of virus (route of injection)	VSV-Ind	VSV-NJ	Purified VSV for serotype:		Purified G for serotype:		VSV-Ind	VSV-NJ		
						Ind	NJ	Ind	NJ				
Ind (10 ⁶)	Live (i.v.)	None		+	+	+	+	+	+	+	+	+	+
NJ (10 ⁶)	Live (i.v.)	None		-	+	+	+	+	+	+	+	+	+
Ind (10 ⁶)	Live (i.p.)	None		+	+	+	-	NT ^c	NT				
NJ (10 ⁶)	Live (i.p.)	None		-	+	+	+	NT	NT				
Ind (10 ⁸)	Live (i.p.)	None		+	+	+	-	NT	NT				
NJ (10 ⁸)	Live (i.p.)	None		-	+	+	+	NT	NT				
Ind (10 ⁶)	Live (i.v.)	Ind (10 ⁶)	Live (i.v.)	+	+	+	+	+	+	+	+	+	+
NJ (10 ⁶)	Live (i.v.)	NJ (10 ⁶)	Live (i.v.)	-	+	+	+	+	+	+	+	+	+
Ind (10 ⁸)	UV-inactivated and CFA (i.p.)	Ind (10 ⁶)	Live (i.v.)	+	+	+	-	+	+	+	+	+	-
NJ (10 ⁸)	UV-inactivated and CFA (i.p.)	NJ (10 ⁶)	Live (i.v.)	-	+	+	+	+	+	+	+	+	+
Ind G	(i.v.)	Ind G	(i.v.)	+	+	+	-	NT	NT	+	+	+	-
NJ G	(i.v.)	NJ G	(i.v.)	-	+	+	+	NT	NT	-	+	+	+
Ind G	CFA (i.p.)	Ind G	(i.v.)	+	+	+	-	+	+	+	+	+	-
NJ G	CFA (i.p.)	NJ G	(i.v.)	-	+	+	+	+	+	+	+	+	+

^a Titers were determined in steps of two; + + +, positivity up to 8 to 11 dilution steps; + +, 5 to 7 steps; +, 2 to 4 steps.

^b See reference 10.

^c NT, Not tested.

reactivity patterns observed against the whole virus when compared with that against purified G may result from the detection of antibodies against accessible internal VSV proteins (matrix or nucleocapsid) in the purified preparation of whole virus (3, 5, 7, 8, 12). Further testing of these antibody responses on purified internal VSV proteins may resolve this question. Alternatively, because G protein still containing traces of detergent was used in the ELISA, some differences could be attributed to better accessibility or to the fact that new epitopes were formed as a consequence of denaturation during isolation and fixation of G molecules onto plastic.

No obvious asymmetric pattern of cross-reactivity has been found with monoclonal antibodies (7, 8, 12). Hybridization of B cells and cloning of hybridomas in vitro, however, may select antibody specificities that differ from those usually seen in vivo.

Interestingly, the asymmetry of cross-reactivities, as seen by ELISA on purified VSV for antisera from mice primed i.v. with 10⁶ live VSV and as seen for sera from mice immunized with various VSV antigen preparations in an ELISA with VSV G, was opposite that which was observed previously for cytotoxic T cells (10). After i.v. infection with live VSV, mice generated primary cytotoxic T cells specific for VSV-Ind that had high cross-reactivities with target cells infected with VSV-Ind and VSV-NJ; in contrast, VSV-NJ-specific cytotoxic T cells lysed VSV-Ind-infected targets to a considerably lesser extent than they lysed VSV-NJ-infected cells (10).

Overall, the findings suggest that after infection with serologically distinct but related VSV serotypes, B cells respond foremost to antigenic determinants that contribute most significantly to protection; only if the antigen is injected together with CFA or when very high doses of virus are used are antibody responses against shared determinants gener-

ated. However, the different asymmetries of the cross-reactivity patterns seen for antibodies and cytotoxic T cells suggest that T and B cells either have different specificity spectra or that they recognize different antigenic epitopes because these epitopes are differentially accessible to T or B cells (4, 16).

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