Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 1257–1261, February 1995
Immunology

Early high-affinity neutralizing anti-viral IgG responses without further overall improvements of affinity

HANS-PETER ROOST*†, MARTIN F. BACHMANN*†, ANDREAS HAAG*, ULRICH KALINKE*, VLADIMIR PLISKA‡, HANS HENGARTNER*, AND ROLF M. ZINKERNAGEL*§

*Institute of Experimental Immunology, Department of Pathology, University of Zürich, Schmelzbergstrasse 12, CH-8091 Zürich, Switzerland; and ‡Department of Animal Science, Swiss Federal Institute of Technology (ETH), CH-8091 Zürich, Switzerland

Communicated by Richard M. Krause, National Institutes of Health, Bethesda, MD, October 14, 1994

ABSTRACT **Affinity maturation of IgG antibodies in adaptive immune responses is a well-accepted mechanism to improve effector functions of IgG within 2 weeks to several months of antigen encounter. This concept has been defined mainly for IgG responses against chemically defined haptens. We have evaluated this concept in a viral system and analyzed neutralizing IgG antibody responses against vesicular stomatitis virus (a close relative of rabies virus) with a panel of monoclonal antibodies obtained early (day 6 or 12) and late (day 150) after hyperimmunization. These neutralizing IgG antibodies recognize a single major antigenic site with high affinities (K_a of 10^8–10^{10} liter·mol^{-1}) and with rapid on-rates already on day 6 of a primary response and with no evidence for further antigen dose- and time-dependent overall improvement of affinity. This type of IgG response is probably representative for viruses or bacterial toxins which are crucially controlled by neutralizing antibodies.**

Studies with chemically defined small antigenic determinants—i.e., haptens—linked to a carrier protein have shown that during the immune response the late antibodies exhibit higher affinities (1) and faster on-rates (1–6) than early IgG antibodies. However, affinity maturation of an IgG antibody response taking more than a week may not be efficient enough against bacterial toxins or those cytopathic viruses where neutralizing antibodies are essential for protection, because too few antibodies may be generated too late (7–9).

Vesicular stomatitis virus (VSV) is closely related to rabies virus and can infect many species; it may cause a paralytic disease after experimental peripheral infection in mice (10, 11). Neutralizing IgG antibody responses specific for the viral glycoprotein of rabies virus or VSV are necessary for and efficient in protecting vertebrate hosts against infection (12–15). Interestingly, naive specific pathogen-free or conventionally kept mice generate T-cell-independent neutralizing IgM antibodies very early after infection, by day 3 or 4 (16, 17); the strictly T-cell-dependent (18) switch to IgG is observed by days 6–8. This represents a truly primary response, since VSV-primed mice exhibit an accelerated IgG response by days 2–4. High neutralizing titers of 10^{-4} to 10^{-5} are reached by days 9–12 after a primary infection and usually stay rather constant for >6 months.

The present study attempted to assess the time- and dose-dependent neutralizing antibody responses against VSV [substrain Indiana (Ind)]. These analyses revealed that neutralizing antibodies recognized only one major antigenic site on the viral glycoprotein. A panel of monoclonal neutralizing antibodies derived from various immunization protocols by varying time and antigen doses were used to measure and compare affinities, on-rates, and neutralizing activities. The means and ranges of these values were already high on day 6 and did not change by more than a factor of 2–3 independent of virus dose and time after infection.

METHODS

Affinity Measurement. Affinity was determined directly from the hybridoma supernatants by an ELISA method (19, 20). Briefly, antibody concentration of the supernatants was determined with subclass-specific anti-mouse IgG antibodies with a mouse myeloma IgG subclass standard. To determine the affinity, ELISA plates were coated with purified VSV at three different densities, and antibody concentrations leading to half-maximal absorbance were determined. By using three different antigen densities in the solid-phase assay, it was possible to extrapolate the antibody input concentration needed for half-maximal binding to a situation where the antibody concentrations and input concentrations were equal (for details, see ref. 20). The affinity of some monoclonal antibodies was also measured in solution by a competition assay (17, 21).

Neutralizing-Activity Measurement. The neutralizing activity was measured and standardized for the antibody concentration of 1 μg/ml (22).

On-Rate Measurement. The second-order on-rate (k_{on}) was determined by measuring the velocity of virus neutralization. Antibody concentrations neutralizing VSV completely in the standard neutralization assay were incubated with VSV(Ind) for 90, 60, 40, 30, 20, 15, 10, or 5 min and plaqued on Vero cells. To simplify the evaluation, conditions were chosen to obtain a pseudo-first-order reaction, by keeping antibody in excess—i.e., antibody concentrations did not change during the measurement. Linear plots were obtained when the logarithm of residual plaque-forming units (pfu) was plotted against the time of coincubation. By linear regression, the slope of this line was evaluated (k_{app}). By dividing k_{app} by the antibody concentration, k_{on} was calculated. To improve the measurements, k_{app} was determined for several antibody concentrations and k_{on} was calculated from the mean.

Epitope Mapping. Epitopes were defined by using mouse or rat monoclonal antibodies on VSV-infected Vero cells. Infected cells were incubated for 30 min with a mixture of competitor mouse monoclonal antibody (IgG1, VI 7) used at near saturation and a panel of mouse IgG2a antibodies that were detected with a labeled IgG2a-specific second antibody. Alternatively, a panel of rat monoclonal IgG antibodies was mixed with competitor murine monoclonal antibody VI 24, 22, or 48 and staining was performed with a rat-specific goat antibody for flow cytofluorometric analysis.

Only those rat and mouse antibodies which were displaced >50% by the competing antibody were considered to be competitively inhibited significantly. To assess competition of a

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: VSV, vesicular stomatitis virus; pfu, plaque-forming units.

†H.-P.R. and M.F.B. contributed equally to this work.

§To whom reprint requests should be addressed.

mouse monoclonal antibody against a rat polyclonal anti-VSV(Ind) serum, the latter was preadsorbed for 18 hr at 4°C on 4×10^7 VSV(New Jersey)-infected Vero cells to remove all crossreactive nonneutralizing antibodies, leaving the neutralizing antibodies in solution. The VSV(Ind)-infected Vero cells were first incubated with an excess of the competitor (monoclonal antibody VI 48) and then incubated with rat polyclonal antiserum, washed, and stained with a fluorescein-labeled goat anti-rat antibody.

Preparation of Fab Fragments. Fab fragments were prepared by digestion with papain (23). Since one antibody molecule with two binding sites leads to two Fab fragments with one binding site, the molar concentration of active sites in the antibody solution before digestion is the same as the concentration of Fab fragments after digestion. Three independent Fab preparations of the same monoclonal antibody (VI 24) were analyzed with identical results.

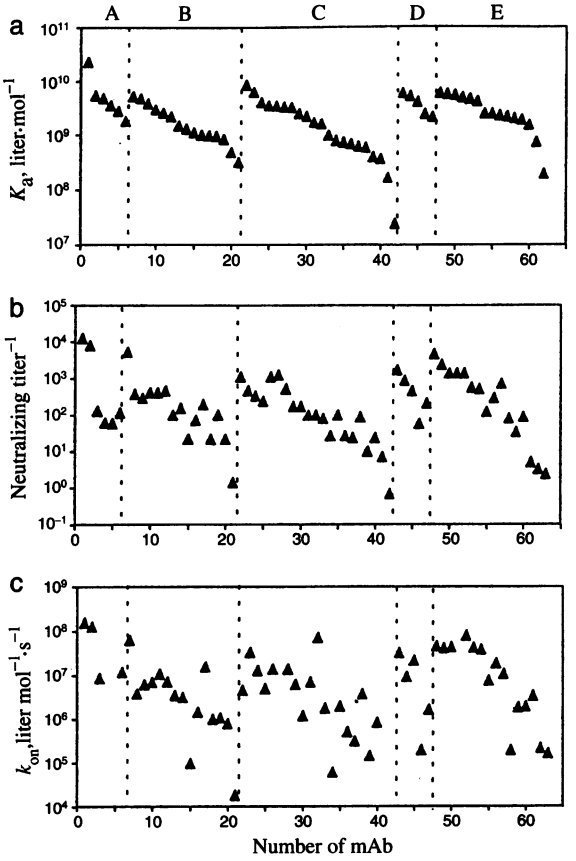
Scatchard Analysis. The following equation was used: $(OD/OD_{max})/\text{antibody concentration} = 1/K_d - (OD/OD_{max})/K_d$. OD and OD_{max} were corrected for background; OD/OD_{max} represents saturation. We plotted in the Scatchard plots saturation/antibody concentration on the y axis vs. saturation on the x axis. We do not know the actual free concentrations of antibody but only the input concentrations. However, from the analysis of the ELISA data with the method described above, which corrects for the proportion of virus-adsorbed antibody, it was evident that only a minor proportion of antibody bound to the coated virus in the plate (data not shown). Thus, we replaced the free antibody concentration by the input antibody concentration.

Affinity Spectra. Affinity spectra show relationships between "binding capacity" (ordinate; expressed by OD values assessed in ELISA) and dissociation constant (abscissa K_d) computed for consecutive subsets of experimental points (for details see ref. 24). Each vertical line represents a single subset. Note that all subsets yield K_a values within a narrow range, thus demonstrating the homogeneity of the corresponding binding sites.

RESULTS

Derivation of Monoclonal Neutralizing Antibodies Against VSV. To obtain a panel of defined monoclonal neutralizing IgG antibodies against VSV(Ind), mice were immunized with 2×10^6 pfu (high dose) or 2×10^3 pfu (low dose) of VSV(Ind) and their spleen cells were fused to P3X63Ag8.653 myeloma cells on day 6 after primary infection, on day 12 (after a single booster immunization on day 9) or on day 150 after regular booster immunizations at intervals of 3 weeks (Fig. 1). Hybridomas were first screened by ELISA for binding of IgG to purified VSV (day 6); since all antibodies specific for VSV glycoprotein also neutralized VSV, the other hybridomas were screened by neutralization (days 12 and 150).

Mapping of the Immunodominant Neutralizing Epitopes. To map antigenic domains, competition assays were performed by using infected cells expressing the neutralizing epitope; this permitted easy separation of bound from nonbound antibodies. A fluorescent second-stage antibody specifically detected mouse or rat monoclonal or polyclonal antibodies which bound after competition with a given mouse monoclonal antibody (Fig. 2). As expected, the higher the neutralizing titers of the antibodies were, the less could their binding be inhibited competitively (data not shown). Almost all antibodies (about 90% of >60 rat or mouse monoclonal antibodies) competed mutually (Fig. 2*a*); these findings thus extended earlier studies with VSV(Ind) (25) showing three partially overlapping epitopes which apparently form one single major neutralizing antigen structure. This conclusion is strongly supported by the fact that a single high-affinity mouse monoclonal neutralizing IgG2a antibody (VI 48) successfully competed with >95% of rat polyclonal neutralizing IgG (Fig.



d

Panel	*n*	Parameter	Arithmetic mean ± SEM	Geometric mean	Median
A (day 6, 2×10^6 pfu)	6	K_a	$(8.0 \pm 3.3) \times 10^9$	4.0×10^9	4.2×10^9
		k_{on}	$(7.8 \pm 3.9) \times 10^7$	3.9×10^7	7.1×10^7
		Neut. titer^{-1}	3530 ± 2260	426	126
B (day 12, 2×10^6 pfu)	15	K_a	$(2.0 \pm 0.4) \times 10^9$	1.5×10^9	1.3×10^9
		k_{on}	$(8.5 \pm 4.1) \times 10^6$	2.3×10^6	3.6×10^6
		Neut. titer^{-1}	560 ± 316	134	174
C (day 12, 2×10^3 pfu)	21	K_a	$(2.0 \pm 0.5) \times 10^9$	3.2×10^9	4.2×10^9
		k_{on}	$(6.5 \pm 1.9) \times 10^6$	2.5×10^6	4.5×10^6
		Neut. titer^{-1}	260 ± 81	119	100
D (day 150, 2×10^6 pfu)	5	K_a	$(4.0 \pm 0.8) \times 10^9$	3.2×10^9	4.2×10^9
		k_{on}	$(1.3 \pm 0.6) \times 10^7$	4.2×10^6	9.6×10^6
		Neut. titer^{-1}	672 ± 300	390	464
E (day 150, 2×10^3 pfu)	15	K_a	$(3.2 \pm 0.5) \times 10^9$	2.5×10^9	2.6×10^9
		k_{on}	$(2.3 \pm 0.6) \times 10^7$	7.1×10^6	1.1×10^7
		Neut. titer^{-1}	792 ± 306	200	283
F (all)	62	K_a	$(3.0 \pm 0.4) \times 10^9$	1.8×10^9	2.3×10^9
		k_{on}	$(1.7 \pm 0.4) \times 10^7$	4.2×10^6	6.3×10^6
		Neut. titer^{-1}	821 ± 260	140	225

FIG. 1. Affinities (K_a, liter·mol^{-1}), neutralizing capacities (titer^{-1}), and on-rates (k_{on}, liter·mol^{-1}·s^{-1}) of monoclonal anti-VSV(Ind) antibodies. The constants measured with each antibody are listed directly above the number of the hybridoma. Hybridoma clones were obtained by fusion of P3X63Ag8.653 myeloma cells to spleen cells taken from BALB/c mice on day 6 after immunization with 2×10^6 pfu (high dose) of VSV(Ind) [clones 1–6 (A)], on day 12 after immunization and booster infection on day 9 with 2×10^6 pfu (high dose) [clones 7–21 (B)] or 2×10^3 pfu (low dose) [clones 22–42 (C)] of VSV(Ind), or 150 days after immunization and booster infections every 3 weeks with 2×10^6 pfu [clones 43–47 (D)] or 2×10^3 pfu [clones 48–62 (E)] of VSV(Ind). (*a*) Affinity (equilibrium association constant, K_a). (*b*) Neutralizing capacity, expressed as standardized neutralizing titer of monoclonal antibodies with a starting concentration of 1 μg/ml. (*c*) Second-order on-rates (k_{on}). (*d*) Statistics of the constants of the individual monoclonal antibody panels (A–E) or of all the antibodies (F).

a

Competing antibodies		Detected antibodies,
mAb	Subclass	no./no. tested
VI 7	IgG1	30/33 mouse IgG2a mAb
VI 24	IgG1	28/31 rat IgG mAb
VI 22	IgG2a	28/31 rat IgG mAb
VI 48	IgG2a	28/31 rat IgG mAb
VI 48	IgG2a	95% of a rat day 8 polyclonal antiserum

b

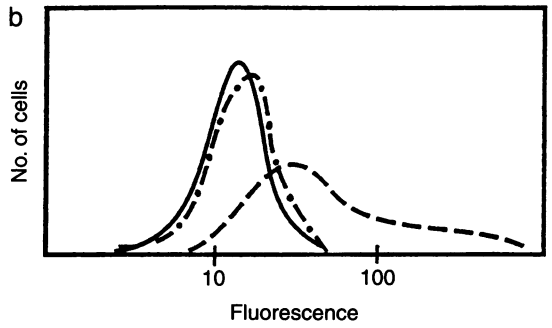


FIG. 2. Determination of the number of neutralizing epitopes on VSV(Ind) by competitive inhibition studies. (*a*) Four mouse monoclonal antibodies (mAbs) were used in excess to compete against a panel of 31 rat or 33 mouse mAbs or a rat polyclonal anti-VSV(Ind) serum obtained on day 8 of a primary infection. Detected antibodies were used at a concentration yielding 90% of maximal surface staining of infected cells; successfully competed versus total number of tested antibodies is indicated. (*b*) Fluorescence distribution of VSV(Ind)-infected Vero cells after incubation with the detected rat polyclonal IgG alone (– – –), the competing mouse mAb VI 48 and the detected rat polyclonal antibodies (–·–·), or the competing mouse mAb VI 48 alone (——).

2*b*). Thus, not only the various monoclonal antibodies but also the major fraction of a rat day 8 polyclonal neutralizing IgG response *ex vivo* were specific for a single major epitope (Fig. 2*b*). The finding that neutralizing antibodies bound to one major antigenic site made possible a comparison of their physicochemical properties.

Measurement of Antibody Affinities and On-Rates. The affinity of monoclonal antibodies was measured in a solid-phase ELISA and on-rates were determined by measuring the velocity of virus neutralization. The comparative analysis of 62 mouse monoclonal neutralizing antibodies (Fig. 3) revealed that (*i*) affinities and on-rates correlated well with specific neutralization titers and (*ii*) there was no significant difference between the average specific antibody titers, affinities, or on-rates of monoclonal antibodies obtained with the various immunization protocols, including day 6 antibodies (Fig. 1). To confirm the affinity measurement with a second, independent method that measures affinity in solution, a competition assay was performed. The apparent affinities of three representative antibodies (VI 10, ELISA $K_{aff} = 3 \times 10^9$ liter·mol^{-1}; VI 24, ELISA $K_{aff} = 4 \times 10^9$ liter·mol^{-1}; VI 41, ELISA $K_{aff} = 1.7 \times 10^8$ liter·mol^{-1}) were thus measured by determining the concentration of viral glycoprotein in solution required for half-maximal competition for antibody binding (21); the apparent affinities were within the same ranges (VI 10, $K_{aff} = 5 \times 10^9$ liter·mol^{-1}; VI 24, $K_{aff} = 5 \times 10^9$ liter·mol^{-1}; VI 41, $K_{aff} = 2 \times 10^8$ liter·mol^{-1}), differing only by a factor of ≤ 2 from the ELISA results, as found previously in other model systems (26, 27).

The correlations between affinity (and on-rate) and neutralizing capacity are not direct proof but are quite compatible with the idea that one antigenic site is being recognized by these IgG antibodies. To establish whether the high-affinity binding detected by ELISA reflected monovalent binding, modified affinity-spectra analysis was carried out over a 10^5-fold range of concentrations (Fig. 4). The results indeed indicate the occurrence of a single population with homogeneous binding as demonstrated by the Hill coefficient close to unity for all investigated monoclonal antibodies (24). Scatchard plot analysis (Fig. 4), revealing a linear relationship, also indicates a lack of cooperativity for the antibody tested (28). These results were supported by the analysis of Fab fragments of one monoclonal antibody (VI 24); comparison of affinities of control undigested monoclonal IgG antibodies and Fab fragments revealed values differing only by a factor of ≤ 2 (Fig. 4).

To confirm these results in a second assay system, Fv fragments consisting of the variable region of the antibody VI 24 linked to a κ light-chain constant (C_κ) domain were generated by genetic engineering (29). These fragments bound to VSV with the same affinity (even under reducing conditions to prevent dimerization of Fv fragments) before or after crosslinking with an anti-C_κ antibody, confirming the results obtained with the Fab fragments in a chemically defined situation (U.K., unpublished data). Therefore, at least this selected, early (day 12) antibody binds to VSV monovalently with high affinity. One of 10 antibodies tested (VI 41) did not exhibit a linear Scatchard plot and therefore probably does not bind to VSV monovalently.

DISCUSSION

We have analyzed numerous monoclonal VSV-specific IgG antibodies that are representative of a complex antiserum. Already on day 6 during a primary response IgG antibodies against the unique neutralizing epitope on the VSV glycoprotein exhibited high affinity with rapid on-rates. The quality of this response did not improve further with respect to average range of affinities, on-rates, and neutralizing capacity in a time- and dose-dependent fashion. How can these findings for a biologically defined epitope that is of key importance for antiviral protection (12, 14, 15) be explained, how represen-

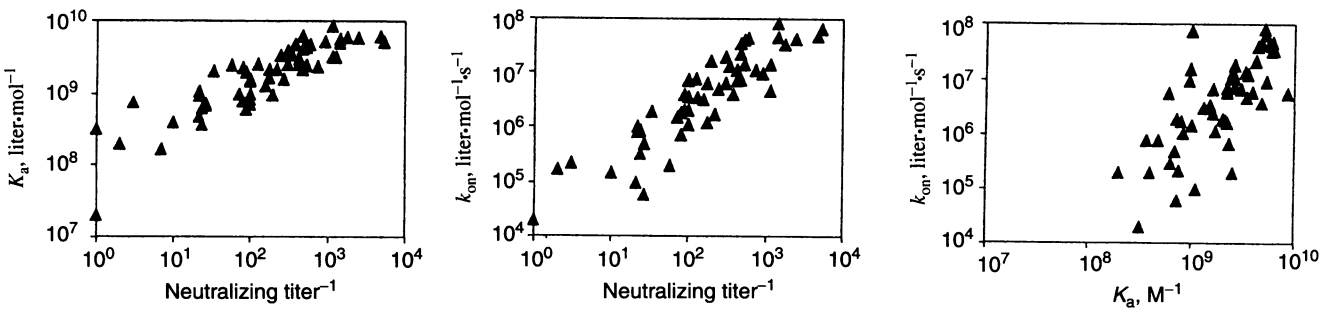


FIG. 3. Correlation between neutralizing capacity and affinity constant (*Left*), neutralizing capacity and on-rate constant (*Center*), and affinity and on-rate constant (*Right*). Constants were determined as described in Fig. 1. All correlations were statistically highly significant, with $P < 0.0001$ (*F* test).

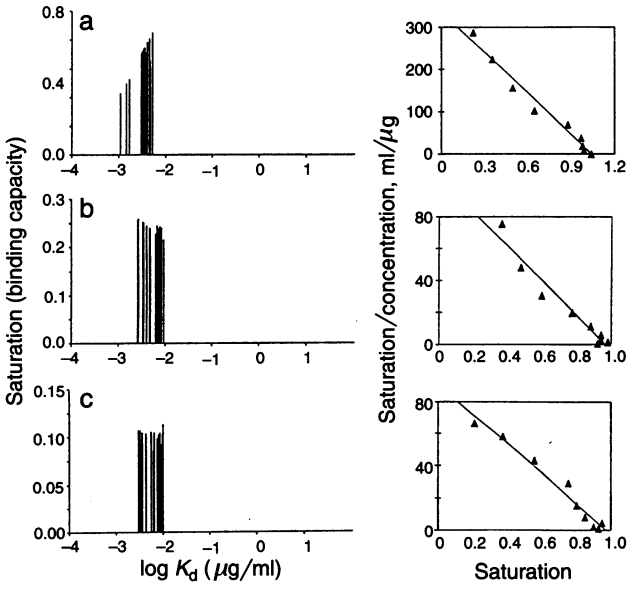


FIG. 4. Binding of two monoclonal antibodies and one Fab fragment to VSV. Shown are affinity spectra obtained within the concentration range used in the binding experiments (10^{-4} to 10 μg of IgG per ml) (*Left*) and Scatchard plots of the same experiments (*Right*). (*a*) Antibody VI 1 ($K_a = 2 \times 10^{10}$ M^{-1}). (*b*) Antibody VI 24 ($K_a = 4 \times 10^9$ M^{-1}). (*c*) Fab fragment of VI 24 ($K_a = 2 \times 10^9$ M^{-1}). In *a*, the ELISA was performed with a goat anti-mouse IgG antibody; in *b* and *c*, the ELISA was performed with a goat anti-mouse κ chain.

tative are these findings, and how can they be compared with those obtained with anti-hapten responses (1, 3–6, 30)?

Limitations of the Methods Used. Affinity measurements in solution by equilibrium dialysis or quenching are applicable for haptens (1) but cannot be performed for neutralizing epitopes on a complex folded protein of 60 kDa. The solid-phase ELISA method employed here (19, 20) is therefore not ideal but has been established and validated by comparing affinities of antibodies against the carcinoembryonic antigen (31). In addition, we confirmed the results by measuring apparent affinities in solution with a competition assay. For many antibodies no cooperativity was observed in our solid-phase ELISA affinity measurements. Experimental work (28) and mathematical modeling (24) of the data confirmed monovalent high-affinity antigen binding (Fig. 4). The data in Fig. 1 indicate no skewing of measured affinity values. In addition, since affinities up to 2×10^{10} liter·mol^{-1} could be measured (see antibody VI 1 in Fig. 1*a*), significant increases in affinities should have been detected and should at least have skewed the overall distribution of affinities toward higher values. This was not found, and therefore the lack of affinity increases seems not to be due to technical limitations of measuring affinities and on-rates (32).

It is important to emphasize that the question whether the monoclonal antibodies bind to VSV with high affinity or avidity is of great interest; but *in vivo*, this distinction between affinity and avidity is probably not very important, since specific B cells are selected by the virus and not by a monovalent antigen and exhibit therefore the same binding properties as in the ELISA (which uses purified virus).

The differences between haptens and VSV may be explained by the antigen organization (i.e., repetitiveness of epitopes) and structure and drastically different antigen kinetics after immunization with haptens vs. virus infections. VSV is a cytopathic, replicating antigen that is controlled initially by interferons and neutralizing antibodies which are generated with high efficiency within a few days after infection. Experiments with inactivated virus and recombinant glycoprotein suggest that an immunization dose of 2×10^6 pfu of live virus corresponds to about 10 μg of virus. In contrast, hapten–carrier antigens in adjuvant usually are used at higher doses, 100–1000 μg. In addition, because of the adjuvant, hapten antigens do not exhibit sharp antigen kinetics; antigen cannot be eliminated efficiently from the depot and therefore antibodies of higher affinity may be gradually selected. In contrast, during infection with a poorly replicating cytopathic virus, antigen concentration is about maximal during the first few days when the immune response is initiated, and antigen is eliminated quickly so that further overall affinity increase is therefore negligible after day 6 [although VSV antigens do persist on follicular dendritic cells (33)]. Nevertheless, even repeated booster infections were not able to force further increase of the average affinity of the antibodies. Memory levels of neutralizing anti-VSV IgG titers are usually between 1:80,000 and 1:320,000, remain stable within factors of 2–4 for >6 months, and do not increase by more than a factor of 4–8 after booster infections compared with the response after a single injection. These findings further support the notion that the overall quality of the neutralizing antibody response is rather independent of the immunization protocol used.

How Could High-Affinity Antibodies to VSV Be Generated So Early? First, it is noteworthy that the early (day 6) monoclonal antibodies were first selected by glycoprotein-specific ELISA, but upon second testing they were all found to be neutralizing. This observation and the evidence that VSV particles behave like a specific T-help-independent antigen (17) for IgM antibodies, as well as the collected sequence information (U.K., unpublished work), suggest that VSV may have evolved to induce neutralizing antibody responses preferentially early and very efficiently against one unique antigenic determinant. Therefore, induction of high-affinity neutralizing antibodies by some viruses may be considerably more efficient than for other antigens, because many viruses preferentially infect antigen-presenting cells (17) and/or trigger B cells directly due to repetitive, organized neutralizing determinants (17). In fact, induction of anti-hapten antibody responses is usually difficult (for exceptions see refs. 34 and 35), since it requires the use of adjuvant and boosting (1, 3, 30).

Second, is the anti-VSV neutralizing antibody specificity encoded by germline genes? We have found that a few neutralizing antibodies use germline genes whereas others show variable numbers of mutations (unpublished observations). Since VSV is not considered to be a natural mouse pathogen, the fact that a few neutralizing antibodies are germline-encoded may indicate that the germline fixation of this specificity may be older than the speciation of the mouse and that VSV has adapted to the available germline gene repertoire of vertebrates in general. Alternatively and speculatively, could the presence of high-affinity antibodies suggest that B cells with the VSV neutralizing specificity somatically mutate randomly very early, before day 6? VSV particles contain highly organized VSV G that optimally crosslinks receptors of B cells to induce them independently of T-cell help (17). Thereby, the frequency of specific B cells increases about 1000-fold within 4–6 days and B cells may hypermutate. For the isotype switch from IgM to IgG, cognate T-cell help is essential (18). When T-cell help is induced by days 4–6, probably only the higher-affinity B cells compete against the high-titered IgM antibodies for the disappearing antigen. In this case, only these high-affinity B cells can be triggered to switch from IgM to IgG by day 6. In contrast to the anti-VSV response, we speculate that in hapten systems (where this T-cell help-independent B-cell proliferation and IgM production is not observed) the early T-independent random hypermutation and the subsequent T-dependent clonal selection cannot be separated.

How General Are the Presented Findings? Extensive qualitative analyses of antibody responses to viruses have not been done. Analysis of secondary hemagglutinin (HA-Sb)-specific monoclonal antibodies isolated from mice on day 24 after

priming with influenza virus and boosting on day 21 revealed examples of somatic point mutations leading to increased avidity (36). Earlier studies involving complex sera described enhanced crossreactivity patterns in the late antibody response to influenza virus, also suggesting avidity changes during the ongoing immune response (37). However, dose- and time-dependent comparisons of antibody qualities have not been made with a large antibody panel. We speculate that the type of response found here for VSV is probably representative for viruses or bacterial toxins that are controlled exclusively or primarily by an early neutralizing antibody response (13). It is likely that slow affinity maturation of antibody responses occurs against other viruses that are initially primarily controlled by cytotoxic T cells and not by antibodies [e.g., lymphocytic choriomeningitis virus (38) and human immunodeficiency virus (39)]. The latter viruses may need to spread for a prolonged time and therefore may have been selected to depend on slow affinity maturation by extensive and prolonged somatic mutation (40) before IgG antibodies reach affinities that are protective *in vivo*.

We thank the many reviewers for discussion and help. We thank Ms. Y. Deflorin for excellent secretarial assistance. These studies were supported by Swiss National Science Foundation Grants 31-32195.91 and 32-36244.92, the Jeantet Foundation, and the Kanton Zürich.

1. Eisen, H. N. & Siskind, G. W. (1964) *Biochemistry* **3,** 996–1008.
2. Anonymous (1986) *Lancet* **ii,** 372–374.
3. Berek, C., Griffiths, G. M. & Milstein, C. (1985) *Nature (London)* **316,** 412–418.
4. French, D. L., Laskov, R. & Scharff, M. D. (1989) *Science* **244,** 1152–1157.
5. McKean, D., Huppi, K., Bell, M., Staudt, L., Gerhard, W. & Weigert, M. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 3180–3184.
6. Foote, J. & Milstein, C. (1991) *Nature (London)* **352,** 530–532.
7. Mims, C. A. (1987) *The Pathogenesis of Infectious Disease* (Academic, London), p. 254.
8. Langman, R. E. & Cohn, M. (1987) *Mol. Immunol.* **24,** 675–697.
9. Zinkernagel, R. M., Hengartner, H. & Stitz, L. (1985) *Br. Med. Bull.* **41,** 92–97.
10. Wagner, R. R. (1987) *The Rhabdoviruses* (Plenum, New York), p. 1.
11. Baer, G. M. & Cleary, W. F. (1972) *J. Infect. Dis.* **125,** 520–527.
12. Bachmann, M. F., Kündig, T. M., Kalberer, C. P., Hengartner, H. & Zinkernagel, R. M. (1994) *J. Immunol.* **152,** 4235–4241.
13. Gobet, R., Cerny, A., Rüedi, E., Hengartner, H. & Zinkernagel, R. M. (1988) *Exp. Cell Biol.* **56,** 175–180.
14. Lefrancois, L. (1984) *J. Virol.* **51,** 208–214.
15. Dietzschold, B., Tollis, M., Lafon, M., Wunner, W. H. & Koprowski, H. (1987) *Virology* **161,** 29–36.
16. Charan, S. & Zinkernagel, R. M. (1986) *J. Immunol.* **136,** 3057–3061.
17. Bachmann, M. F., Hoffmann Rohrer, U., Kündig, T. M., Bürki, K., Hengartner, H. & Zinkernagel, R. M. (1993) *Science* **262,** 1448–1451.
18. Leist, T. P., Cobbold, S. P., Waldmann, H., Aguet, M. & Zinkernagel, R. M. (1987) *J. Immunol.* **138,** 2278–2281.
19. Beatty, J. D., Beatty, B. G., Vlahos, W. G. & Hill, L. R. (1987) *J. Immunol. Methods* **100,** 161–172.
20. Beatty, J. D., Beatty, B. G. & Vlahos, W. G. (1987) *J. Immunol. Methods* **100,** 173–179.
21. Rath, S., Stanley, C. M. & Steward, M. W. (1988) *J. Immunol. Methods* **106,** 245–249.
22. Roost, H. P., Charan, S. & Zinkernagel, R. M. (1990) *Eur. J. Immunol.* **20,** 2547–2554.
23. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1987) *Current Protocols in Molecular Biology* (Wiley, New York), Vol. 1.
24. Pliska, V., Heiniger, J., Müller-Lhotsky, A., Pliska, P. & Ekberg, B. (1986) *J. Biol. Chem.* **261,** 16984–16989.
25. Lefrancois, L. & Lyles, D. S. (1982) *Virology* **121,** 157–167.
26. Frankel, M. E. & Gerhard, W. (1979) *Mol. Immunol.* **16,** 101–106.
27. Azimzadeh, A., Pellequer, J. L. & Van Regenmortel, M. H. V. (1992) *J. Mol. Recognit.* **5,** 9–18.
28. Azimzadeh, A. & Van Regenmortel, M. H. V. (1991) *J. Immunol. Methods* **141,** 199–208.
29. Traunecker, A., Oliveri, F. & Karjalainen, K. (1991) *Trends Biotechnol.* **9,** 109–113.
30. Rajewsky, K., Förster, I. & Cumano, A. (1987) *Science* **238,** 1088–1094.
31. Wagener, C., Clark, B. R., Rickard, K. J. & Shively, J. E. (1983) *J. Immunol.* **130,** 2302–2307.
32. Dekker, E. L., Porta, C. & Van Regenmortel, M. H. V. (1989) *Arch. Virol.* **105,** 269–286.
33. Bachmann, M. F., Kündig, T. M., Odermatt, B., Hengartner, H. & Zinkernagel, R. M. (1994) *J. Immunol.* **153,** 3386–3397.
34. Dintzis, H. M., Dintzis, R. Z. & Vogelstein, B. (1976) *Proc. Natl. Acad. Sci. USA* **73,** 3671–3675.
35. Dintzis, R. Z., Tyler, A. N. & Dintzis, H. M. (1983) *J. Immunol.* **131,** 2196–2203.
36. Clarke, S., Rickert, R., Wloch, M. K., Staudt, L., Gerhard, W. & Weigert, M. (1990) *J. Immunol.* **145,** 2286–2296.
37. Fazekas de St. Groth, S. (1967) *Cold Spring Harbor Symp. Quant. Biol.* **32,** 525–536.
38. Lehmann-Grube, F. (1971) *Virol. Monogr.* **10,** 1–173.
39. Rosenberg, Z. F. & Fauci, A. S. (1989) *Adv. Immunol.* **47,** 377–431.
40. Mims, C. A. & Tosolini, F. A. (1969) *Br. J. Exp. Pathol.* **50,** 584–593.