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Neutralizing Antiviral Antibody Responses

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I. Introduction

Antibody responses have been studied in infectious diseases, particularly against toxins isolated from pathogenic bacteria. Indeed, antibody quality requirements for protection against diphtheria toxin were among the first to be elucidated (Von Behring and Kitasao, 1890; Ehrlich, 1906; Jerne, 1951). However, the inability to purify these toxins and the complexity of the biological material forced immunologists to use chemically pure substances in order to quantitatively study antigens and their reactions with antibodies (Evans, 1943). Therefore, immunochemists used haptens, usually small phenyl groups, that were easily synthesized in various configurations and could be linked to large proteins (Heidelberger, 1956; Eisen and Siskind, 1964). Because of their small size they could pass through the then-available dialysis bags to measure physicochemical parameters of antibody/antigen interactions and define binding affinities and specificities. Only recently has it become possible to express and purify biological substances sufficiently not only to repeat some of these analyses *in vitro* but also to correlate such measurements with protection against disease *in vivo* (Fazekas de St. Groth and Webster, 1961; Staudt and Gerhard, 1983; Webster and Rott, 1987; Laver *et al.,* 1990; Bachmann *et al.,* 1997b).

Individuals with defective antibody responses, such as patients with Bruton's disease or other antibody defects, are more susceptible to infections (Wilfert *et al.,* 1977; Graham *et al.,* 1983; Englund *et al.,* 1998), as are mice devoid of B cells, such as the μMT mouse (Good and Zak, 1956; Kitamura *et al.,* 1991; Bründler *et al.,* 1996). Protective or neutralizing antibody responses, particularly against acute cytopathic agents, are among the most critical defense mechanisms (Sabin, 1981; Steinhoff *et al.,* 1995; Zinkernagel *et al.,* 1996). These responses represent the result of a long co-evolution between the host and various infectious agents. However, because of the shorter generation time and greater numbers, adaptation of infectious agents is considerably faster than that of the host. This review attempts to summarize from an evolutionary point of view evidence that neutralizing antibodies (nAbs) are key to protecting the species against many viruses, particularly cytopathic ones. Such viruses have the potential to upset

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the balance between host and pathogen; therefore, an equilibrium must have evolved to enable the survival of both. This balance is maintained by the ability of the host to mount rapid neutralizing antibody responses which is achieved by directly encoding virus-specific neutralizing antibodies in the germline. Thus, it is mandatory to assess the protective capacity of antibodies by *in vivo* adoptive transfer experiments or by *in vitro* neutralization assays, rather than measuring antigen binding in an enzyme-linked immunosorbent assay (ELISA).

Interestingly, although not surprisingly, there is a certain geography of the antibody response: local IgA in the mucosa, systemic short-lived IgM and long-lived IgG in blood. In addition, antibodies not only neutralize viruses and thereby prevent infection but also trap virus in the spleen and prevent it from reaching the blood. They thereby enhance immune responses in general and B cell responses in particular. This review also presents evidence that, in general, B cells are not negatively selected and are largely regulated by antigen structure and availability of cognate T help. We also review evidence supporting the notion that B cell memory, in the form of high neutralizing antibody serum levels, is the main contributor to immunological memory. Finally, both general and specific examples of neutralizing antibody responses are used to illustrate rules that govern B cell responses. These rules are also discussed with respect to their role in autoimmunity and how they can be exploited for vaccines.

II. Antiviral Antibody Responses

During their development in the bone marrow, B cell precursors undergo sequential recombination-activating gene (RAG)-mediated rearrangements of D to J and V to DJ elements of the immunoglobulin (Ig) heavy-chain variable region (V_H) and V to J elements of the Ig light-chain variable region (V_L) (Tonegawa, 1983). Expression of functional Ig heavy and light chains promotes the transition of precursor B cells to small IgM/IgD-positive mature B cells. Autoreactive antibody specificities generated by random rearrangement are either deleted (Nemazee and Buerki, 1989; Nemazee and Buerki, 1989; Nemazee *et al.,* 1991; Hartley *et al.,* 1993) or edited by secondary V_L and possibly V_H replacements (Gay *et al.,* 1993; Radic *et al.,* 1993; Tiegs *et al.,* 1993; Chen *et al.,* 1995, 1997; Lang *et al.,* 1996; Pelanda *et al.,* 1997; Fang *et al.,* 1998; Nemazee, 2000). B cells that emigrate from the bone marrow and populate secondary lymphoid organs constitute the primary B cell repertoire. The restricted number of V, D, and J gene segments that can be encoded in the genome limits the size of the potential repertoire. However, diversity of the preimmune repertoire of mice and humans can be further expanded by hypermutation, whereas other species, such as rabbits, sheep, pigs, and cattle, use gene conversion (Diaz and Flajnik, 1998). It has recently been suggested that, together with hypermutation, secondary Ig gene rearrangements may also contribute to repertoire diversification (Han

et al., 1997; Papavasiliou *et al.,* 1997; Pelanda *et al.,* 1997; Hertz *et al.,* 1998; Meffre *et al.,* 1998; Lopez-Macias *et al.,* 1999; Wilson *et al.,* 2000b). However, whether this considerably slower process confers a survival advantage to the host remains to be formally demonstrated.

A. V(D)J Rearrangements in Antiviral Antibodies

The primary repertoire of vesicular stomatitis virus (VSV)-specific antibodies was analyzed by isolating hybridomas 4 and 5 days after infection (Kalinke *et al.,* 1996a). Surprisingly, 87% of clones analyzed used V genes belonging to the V_HQ52 and $V_\kappa 19$–28 families. Despite their polyclonal origin, the majority of these clones expressed identical V_H and V_L germline segments. Because all these antibodies failed to bind and neutralize the 51.12 VSV variant (which was selected in the presence of one particular $V_HQ52/V_\kappa 19$–28-positive antibody), they all appear to bind to the same subsite within the major antigenic site of the glycoprotein of VSV (VSV-G). Analysis of the primary response to influenza virus also revealed a restricted V_κ usage, with the predominance of a segment belonging to the $V_\kappa 8$ gene family (Clarke *et al.,* 1990b). In contrast, the heavy-chain variable region genes displayed much greater diversity, with several V_H families being used.

In contrast to primary response antibodies, hybridomas isolated from secondary and hyperimmune anti-VSV responses expressed a more diverse set of V genes belonging to the V_HJ558, V_H7183, and V_HQ52 families (Kalinke *et al.,* 1996a). The majority of these antibodies were shown to bind a different subsite of the major antigenic site of VSV-G than the primary response antibodies. Thus, fine specificity diversification of secondary and hyperimmune responses was achieved by newly appearing V gene combinations. Similar findings were obtained from analysis of the secondary response to influenza virus where V region usage differed considerably from the primary response (Clarke *et al.,* 1990a).

B. The Role of Hypermutation in the Generation of Virus-Neutralizing Antibodies

Interestingly, all anti-VSV mAbs isolated up to day 6 post infection, irrespective of which genetic elements they express, were devoid of somatic hypermutation (Kalinke *et al.,* 1996a). This indicates the presence of VSV specificities in the mouse germline antibody repertoire. This was confirmed by the isolation of VSV-neutralizing antibody fragments from phage display libraries generated from naive mice (A. Lamarre, unpublished results). These findings are remarkable because VSV is a new-world virus of ruminants while *Mus musculus* is an old-world rodent. Therefore, the presence of VSV-specific, high-affinity antibodies in the germline cannot be a result of recent co-evolution; instead, this suggests that, in general, cytopathic viruses have been selected to fit the available antibody repertoire rather than vice versa (Roost *et al.,* 1995; Bachmann *et al.,* 1997b).

Similarly, anti-influenza primary response antibodies were largely devoid of somatic mutations (Clarke *et al.,* 1990b). In contrast to primary antibodies, secondary and hyperimmune mAbs did display hypermutations (Clarke *et al.,* 1990b; Kalinke *et al.,* 1996a). Nevertheless, as with the primary V_HQ52/V_κ19–28-positive antibodies, hypermutated anti-VSV antibodies were not able to neutralize the virus variant 51.12. However, compared to the germline antibodies, hypermutated antibodies had approximately a 50-fold increase in their ability to neutralize wild-type VSV (Kalinke *et al.,* 1996a). Therefore, hypermutation did not alter antibody specificity, as far as can be judged by binding competition assays, but it did improve binding quality and neutralizing capacity.

To verify these conclusions, another more heterogeneous group of VSV-neutralizing antibodies typical of secondary and hyperimmune responses was analyzed (Kalinke *et al.,* 2000). Although these antibodies expressed numerous different light chains, they all used the same V_H germline segment belonging to the V_H7183 family in combination with the J_H2 segment. Again, all hyperimmune antibodies contained hypermutations. Despite most of the hypermutated antibodies not being clonally related, some of them showed the same single somatic amino acid substitutions in CDR1 and CDR2. Because no virus variant resistant to neutralization by all V_H7183/J_H2-positive antibodies could be generated, they apparently bound different subsites within the major antigenic site of VSV-G.

To analyze the impact of the common amino acid substitutions on virus binding and neutralization, the secondary response antibody VI24 of the V_H7183/J_H2 group was expressed as a monovalent, recombinant, single-chain antibody consisting of the V_κ and V_H regions linked to a C_κ domain (Kalinke *et al.,* 2000). This antibody fragment expressed four somatic amino acid exchanges in V_H, whereas V_L was germline. The antibody fragment was mutated back to a germline configuration and mutations were introduced in V_H encoding for one or both of the common amino acid substitutions in CDR1 and CDR2. The binding of the hypermutated VI24 antibody fragment was about 50- to 100-fold better than the germline antibody fragment. Expression of the CDR2 amino acid substitution alone improved binding by about 10-fold, whereas the CDR1 substitution did not affect binding. Antibody fragments were dimerized using an anti-C_κ antibody. Dimerization of the VI24 antibody fragment did not improve its binding, whereas binding of the dimerized germline antibody fragment improved by more than 10-fold. Thus, avidity effects did not contribute to the binding of the hypermutated antibody, whereas they seemed to have a major impact on the binding of the germline antibody fragment.

Analysis of the second group of antibodies confirmed the previous observation that hypermutation was no prerequisite for virus neutralization. However, hypermutation improved the binding and the neutralization of VSV *in vitro* up to a level where avidity effects do not seem to further contribute to the quality of antibody binding.

C. Additional Mechanisms of Repertoire Diversification

Although hypermutation remains the principal mechanism of antigen-driven expansion of the primary B cell repertoire, other mechanisms have also been implicated in this process. The extent of diversification generated through secondary Ig gene rearrangements was assessed by analyzing antiviral antibody responses in the quasi-monoclonal (QM) mouse (Lopez-Macias *et al.,* 1999). The QM mouse was generated by site-directed insertion of a rearranged VDJ region specific for the hapten (4-hydroxy-3-nitrophenyl) acetyl (NP) into the endogenous J_H locus (Cascalho *et al.,* 1996). The other heavy-chain allele was inactivated by deletion of all J_H segments. In addition, QM mice have been crossed to $J\kappa^{-/-}$ mice; therefore, theoretically only NP-specific λ^+ antibodies should be generated. However, QM mice infected with VSV, lymphocytic choriomeningitis virus (LCMV) or poliovirus could mount antiviral nAbs, albeit generally with delayed kinetics, and reached titers of sufficiently high levels to confer protection against lethal viral disease. The antiviral antibodies were generated by the replacement of the targeted V_H region with endogenous V_H elements located upstream of the transgene by a process termed *receptor editing* (Gay *et al.,* 1993; Radic *et al.,* 1993; Tiegs *et al.,* 1993; Chen *et al.,* 1995, 1997; Lang *et al.,* 1996; Pelanda *et al.,* 1997; Fang *et al.,* 1998; Nemazee, 2000). These experiments illustrate the potential of secondary rearrangements in the diversification of a very limited antibody repertoire and suggest that this mechanism might also play a role in expanding the normal B cell repertoire. A recent study showed that mice lacking κ light chains, the co-stimulatory molecules CD19 and CD22, or the signaling molecule Btk were not more susceptible than control mice to VSV or LCMV infections, again demonstrating the plasticity of the B cell repertoire (Fehr *et al.,* 2000).

D. Affinity, Avidity, and Concentration Parameters of Protection

Molecular mechanisms of *in vivo* virus neutralization remain largely undefined. However, several studies have attempted to address the quantitative as well as the qualitative requirements of neutralizing mAbs for *in vivo* protection in passive transfer experiments. For determination of the *in vivo* protective capacity of VSV nAbs, SCID (severe combined immuno deficiency) mice devoid of B and T cells were passively immunized with various mAbs and challenged with VSV; 4 days later the brains of surviving mice were assessed for the presence of virus. Secondary and hyperimmune antibodies bearing hypermutations protected at concentrations between 1 and 3 μg/ml (Bachmann *et al.,* 1997b; Kalinke *et al.,* 2000). One of the primary response antibodies devoid of hypermutations protected at a similar concentration (4 μg/ml), whereas all others protected at concentrations $\geq$15 μg/ml (Kalinke *et al.,* 2000). Thus, most primary response

monoclonal antibodies alone protected from a lethal VSV infection at about a 10-fold higher concentration than secondary and hyperimmune antibodies, but polyclonal sera were comparably efficient, early, and late.

Since at the peak of the Ig response theoretically up to 10% of the total Ig can be antigen specific (Bachmann *et al.,* 1994b; Funk *et al.,* 1998), protective serum levels can be rapidly obtained by germline antibodies. During later time points, when a lower percentage of serum Ig is antigen specific, an increased protective capacity generated through hypermutation would be advantageous.

The VSV protection data are in accordance with the prophylactic antibody dose against numerous viruses, including rabies virus, Ebola virus, coronavirus, and mouse influenza virus (3 μg/ml, 5–15 μg/ml, 5–25 μg/ml, and 1–20 μg/ml, respectively) (Dietzschold *et al.,* 1990; Lamarre and Talbot, 1995; Mozdzanowska *et al.,* 1997; Wilson *et al.,* 2000a). Surprisingly, at a concentration of 30 μg/ml, some Ebola virus-specific antibodies protected 100% of experimental animals even when treatment was started one day post virus exposure. Post-exposure protection of hamsters against rabies virus was also reported using a human G-protein-specific mAb (Dietzschold *et al.,* 1990).

The role of Fc-mediated functions and bivalency in protection against virus infection has also been investigated in several animal models. Protection from lethal murine coronavirus infection could be observed when $F(ab')_2$ or Fab antibody fragments were passively transferred prior to viral challenge (Lamarre and Talbot, 1995). However, the protective capacity of Fab fragments was greatly reduced compared with bivalent molecules despite similar *in vivo* half-lives. The reduction in protective capacity of monovalent antibody fragments compared with $F(ab')_2$ fragments correlated with a 14-fold decreased affinity constant. These results suggest that the Fc fragment and bivalency are not absolutely required for *in vivo* protection but that avidity might influence protective capacity. Similar conclusions were reached when recombinant single-chain antibody fragments were used in protection experiments, although the extremely short *in vivo* half-lives greatly reduce their efficacy (Kalinke *et al.,* 1996b; Lamarre *et al.,* 1997).

III. Mechanisms of Neutralization by Antibodies

Studies using various viral infection models have revealed several mechanisms by which antibodies are capable of neutralizing virus. This is not unexpected given the complexity of virus–antibody interactions, alone or together with host cells.

A. Mechanisms of Neutralization

While opsonization by antibodies seems to be the major protective mechanism against bacterial infection, prevention of attachment to, and therefore

infection of, cells is probably the major antibody-mediated effector mechanism against viruses. However, such a general statement does not do justice to the many studies on the numerous potential effector mechanisms of nAbs. Mechanisms of neutralization have been extensively reviewed previously (Della-Porta and Westaway, 1978; Dimmock, 1984, 1993; Iorio, 1988; Bachmann and Zinkernagel, 1997; Stewart and Nemerow, 1997; Burton *et al.,* 2000) and so will not be discussed here again in detail; nevertheless, we would like to emphasize how little we really know about these mechanisms. Various categories of neutralizing antibody activities have been described: (1) prevention of adsorption; (2) impairment of the function of viral surface proteins necessary for productive infection following virus entry (Levine *et al.,* 1991); (3) intracellular neutralization via a pH-dependent inhibition of virus fusion to vesicles or phagosomes; and (4) aggregation of virus, or pseudo-neutralization, resulting in the reduction of replicating viral particles without necessarily changing the infectivity of each single particle (Wallis and Melnick, 1967). Interestingly, neutralization of some viruses requires binding of antibodies to more than half of the available surface determinants in order to prevent virus attachment and entry into cells. This has been shown to be required for neutralization of rhabdoviruses and may hold true for many other enveloped viruses expressing only one major neutralizing determinant (Flamand *et al.,* 1993; Kalinke *et al.,* 1996b; Bachmann and Zinkernagel, 1997; H.P. Roost, unpublished). For other viruses, such as poliovirus (Dulbecco *et al.,* 1956; Mandel, 1976; Emini *et al.,* 1983) or influenza virus (Lafferty, 1963), only a few antibody molecules (3–5) appear to suffice to inhibit infection. Such differences clearly show that distinct mechanisms may be employed by antibodies for neutralization. Whether neutralization of poliovirus requires a one- or multi-hit mechanism is still debated (Dulbecco *et al.,* 1956; Dimmock, 1984, 1993). We believe the one-hit neutralization model may be an oversimplification and is probably not a general rule. Bacterial phages have also been used to examine antibody–virus interactions; however, because phages usually have only a few possible attachment sites, contrary to animal viruses, the results may not readily be transferable. Despite effective neutralizing properties, antibodies may also enhance infection of cells bearing Fc receptors (Porterfield, 1986), including macrophages and dendritic cells, and potentially B cells, T cells, and endothelial cells (Halstead, 1988; Lewis *et al.,* 1988; Mascola *et al.,* 1993; Battegay *et al.,* 1993a).

Virus nAbs can be measured *in vitro* by assays where either plaque formation by cytopathic viruses or focus formation by noncytopathic viruses is inhibited. ELISA binding assays are less reliable in measuring nAbs except when purified intact viral particles are used. Indeed, antibodies that display neutralizing capacity bind intact viral particles, particularly when only one envelope protein is expressed such as for rhabdoviruses (Bachmann *et al.,* 1994c).

In addition to the ability to neutralize virus on their own, antibodies have also been shown to act in conjunction with complement. The role of complement activation in enhancement or prevention of infection has been well documented, at least for some viruses (Cooper and Nemerow, 1984; Ochsenbein *et al.,* 1999b). In fact, complement consumption has often been used as a readout for the presence of antiviral antibodies; however, it does not necessarily indicate a role for complement in protection nor does it correlate with neutralizing capacity. Inhibition of hemagglutination correlates better with neutralization, although these two assays do not measure the same parameter. In addition, the sensitivity of these assays is quite distinct (Bachmann *et al.,* 1999) and therefore care must be taken when interpreting these correlations.

In conclusion, antibody-dependent neutralization mechanisms are probably largely due to blocking effects, but other mechanisms may apply. However, whether or not mechanisms observed solely *in vitro* are relevant in the living host remains to be further evaluated.

B. Structural Studies

Recent studies have provided insight into the structural aspects of antibody inhibition of virus attachment to cells, specifically in the case of influenza, poliovirus, rhinovirus, adenovirus, and foot-and-mouth disease virus (reviewed in Stewart and Nemerow, 1997). In the case of influenza virus, the binding surface between Fab fragments or intact Ig and hemagglutinin occupies a significantly larger area than the area covered by sialic acid moieties during hemagglutination, by the order of about 1000 A^2 versus 300 A^2 (Stewart and Nemerow, 1997). In the case of human rhinoviruses, comprising many serotypes, ICAM-1 seems to be the major receptor for cellular entry. It was previously thought that nAbs bind to the outer rim of the canyon formed on the virus surface, thus preventing entry of the receptor. However, recent structural evidence has challenged this concept by showing that Fab fragments can actually penetrate the binding site (Smith *et al.,* 1996). Studies with foot-and-mouth disease virus have shown that antibodies can induce a conformational change in the virus surface protein, therefore preventing exposure of the fusogenic domain normally triggered by receptor binding. Similar mechanisms have been postulated for poliovirus, human immunodeficiency virus (HIV), and other viruses. While mechanisms of neutralization that interfere with events occurring after attachment to the host cell have been postulated and experimentally illustrated, structural analyses have not been possible so far.

Taken together, these structural studies have revealed that nAbs either directly block receptor binding or trigger structural changes that may influence cellular entry. Whether or not these interactions occur as such under physiological conditions *in vivo*, particularly in a serum-rich environment, remains to be shown. Structural mechanisms of neutralization of viruses that require complex

stepwise structural changes in order to achieve infection, such as HIV (Robinson and Mitchell, 1990; Poignard *et al.,* 1996), may not be so simple.

IV. The Role of Complement, Natural Antibodies, and Fc-Receptors in Antiviral Responses

A. Natural Antibodies

Natural or spontaneous antibodies are present in the sera of nonimmunized humans and mice (Avrameas, 1991; Herzenberg and Kantor, 1993; Coutinho *et al.,* 1995). Natural antibodies are mainly of IgM isotype, but IgG and IgA have also been observed. Most natural antibodies are polyspecific, although antibodies reacting with a single antigen are also present. $CD5^+$ peritoneal B-1 cells have been shown to be a main source of these polyreactive antibodies, although $CD5^-$ B-2 cells may also participate in the production of the natural antibody repertoire (Casali and Notkins, 1989; Herzenberg and Kantor, 1993). Natural antibodies are encoded by germline genes without, or with very few, somatic mutations. The stimulus for secretion of natural antibodies is largely unknown. It has been shown that B cells synthesizing natural antibodies are also present in newborn, nude, germ-free, and antigen-free mice with similar reactivity patterns when analyzed in a quantitative immunoblot assay (Bos and Meeuwsen, 1989; Haury *et al.,* 1997). A possible explanation for the apparent antigen-independent stimulation of natural antibody-secreting B cells may be that such B cells can normally secrete low levels of Ig or can be triggered by cross-reactive self-antigens.

It has been proposed that natural antibodies may play a role in mediating immunoregulatory functions in the hypothetical idiotypic–anti-idiotypic network theory (Jerne, 1984; Coutinho, 1989) or in autoimmune diseases such as systemic lupus erythematosus (SLE) (Cohen, 1986; Tlaskalova-Hogenova *et al.,* 1992). However, their main physiological function seems to be in early resistance against infections. Natural antibodies together with other components of innate immunity belong to the first line of defense against microbial infections (Boes *et al.,* 1998b; Ochsenbein *et al.,* 1999a; Baumgarth *et al.,* 2000). A role for natural antibodies has also been shown in protection against viral infection through multiple mechanisms. First, highly specific (serotype-specific) IgM antibodies can directly neutralize VSV (Gobet *et al.,* 1988; Ochsenbein *et al.,* 1999a). This normally occurs during hematogenic spread of the virus, although there are some indications in mice that natural IgM antibodies can also be transported to mucosal surfaces and protect locally against influenza virus infection (Baumgarth *et al.,* 2000). Second, because IgM is a pentamer it can potentially bind to ten antigenic determinants per molecule and therefore form large antigen–antibody complexes that would be more efficiently retained

in the spleen (Lutz *et al.,* 1987). This process controls spreading of cytopathic infectious agents through the blood, as shown for VSV, and prevents infection of vital target organs (Ochsenbein *et al.,* 1999a). In addition, the observation that antibody-deficient mice show an increased susceptibility to viruses, such as poliovirus, that must distribute to target organs via blood circulation has provided indirect evidence for such a role (Lachmann and Davies, 1997). Third, IgM is a potent activator of complement (Fearon and Locksley, 1996; Carroll, 1998). Triggering of the complement cascade can directly lead to lysis of invading bacteria or provide protection against viral infections. The efficiency of this protection, however, may be limited due to the fact that NA titers are rather low. If the dose of the cytopathic infectious agent that reaches the systemic circulation is too high, natural antibodies, as a first line of defense, are overrun and specific nAbs have to be elicited quickly and efficiently to prevent infection of vital target organs.

B. Complement

Complement components are involved in host protection against viral infection either directly or indirectly through interaction with virus-infected cells (Cooper, 1991). Enhancement of the neutralization capacity of antibodies by complement components may occur by coating of the virion with C3 and probably to a lower extent with C4 components. This was shown in very early experiments in avian infectious bronchitis where a complete disassembly of the virus was described (Berry and Almeida, 1968). In contrast, *in vitro* experiments using VSV showed that the active cleavage product C3b incorporated into the viral envelope prevented infection of target cells without actually destroying the viral particles (Beebe and Cooper, 1981). Enhancement of antibody-mediated neutralization by complement was recently confirmed *in vivo* with a panel of VSV-specific monoclonal antibodies (Ochsenbein *et al.,* 1999b).

Another way by which direct interaction of complement with virus could enhance neutralization is through the very efficient targeting of complement-coated viral particles to complement receptor (CR)-expressing cells. This effect has been documented for CD21 (CR2)- and CD35 (CR1)-expressing B cells and follicular dendritic cells (FDCs) whereby opsonization of the antigen by complement enhances antigen targeting, leading to more efficient antigen presentation and germinal center (GC) formation (Tew *et al.,* 1990; Carroll, 1998). More recently, a direct enhancement of B-cell receptor (BCR) signal transduction has also been described. This phenomenon was called *dual antigen recognition* because of the simultaneous recognition of antigen by the BCR and complement-bound antigen by CR2 (van Noesel *et al.,* 1993; Fearon and Carter, 1995). Following binding of C3b and C3d, CR2 forms a complex with CD19 and TAPA-1 which induces cross-linking of the BCR with its co-receptors and may thereby lower the threshold for B cell activation. These mechanisms have been shown to be important after immunization with various model antigens.

For example, hen egg lysozyme (HEL) coupled to C3d is 1000- to 10000-fold more immunogenic than HEL alone (Dempsey *et al.,* 1996). Similarly, natural antibodies and complement were shown to be important for the induction of other T-dependent B cell responses in complement-deficient (Ahearn *et al.,* 1996; Molina *et al.,* 1996; Fischer *et al.,* 1998) and soluble IgM-deficient mice (Ehrenstein *et al.,* 1998; Boes *et al.,* 1998a). In contrast, immunization of complement-, complement receptors-, or CD19-deficient mice with different replicating viruses resulted in the generation of neutralizing antibody comparable to controls (Fehr *et al.,* 1998b; Ochsenbein *et al.,* 1999b). This discrepancy suggests that lowering the threshold for B cell activation is of importance mainly for T-dependent antibody responses to limiting amounts of nonreplicating antigens. However, the generation of these T-dependent antibody responses requires at least 6 to 8 days (Oxenius *et al.,* 1998c); therefore, early T-independent (TI) antibody responses are crucial for control of rapidly spreading viruses. Marginal zone macrophages have long been recognized as being important for the generation of TI antibody responses to bacterial antigens (Amlot *et al.,* 1985; Buiting *et al.,* 1996). Recently, analysis of TI-neutralizing antiviral IgM responses also revealed an important role for targeting complement-opsonized virus to marginal zone macrophages expressing CR3 and CR4 (Ochsenbein *et al.,* 1999b). The concentration and localization of antigen were crucial for the induction of early TI antibody responses. Taken together, natural antibodies recognize the pathogen in the circulation, activate the complement cascade, and thereby target the antigen to the splenic marginal zone. This allows cross-linking of BCRs and results in early TI production of neutralizing IgM antibodies (Ochsenbein and Zinkernagel, 2000).

The interaction of complement with virus-infected cells has been studied for several viruses (Welsh *et al.,* 1975; Mills *et al.,* 1979; Beebe and Cooper, 1981; Cooper and Nemerow, 1983, 1984; Cooper, 1991) and appears to be particularly relevant for noncytopathic viruses such as measles virus or Epstein-Barrvirus (EBV). Both viruses activate the human complement cascade via the alternative pathway, leading to lysis of infected cells (McConnell *et al.,* 1978; Sissons *et al.,* 1980). This lytic effect is further increased by antiviral IgG antibodies that probably bind to C3b and thereby increase its resistance to inactivation (Fries *et al.,* 1984; Reiter and Fishelson, 1989). This allows the generation of C5 convertase and further increases lysis of target cells. However, activation of the complement cascade and generation of the lytic complex are counterbalanced by cell repair mechanisms that protect the integrity of nucleated cells through the shedding of vesicles containing pieces of damaged membrane.

An important role for the complement system in antiviral protection is further suggested by the fact that several viruses have evolved strategies to evade complement-mediated lysis either by using complement receptors and complement control proteins as viral receptors or by producing complement blocking or modulating molecules (Frade *et al.,* 1985; Lachmann and Davies, 1997). For

example, HIV has been shown to carry the complement control proteins CD46 and CD59 in its envelope (Saifuddin *et al.,* 1997).

C. Fc-Receptors

While complement and its receptors can interact with pathogens through natural antibodies, thus providing host protection, FcR on immune cells interact with IgG, triggering effector functions and inflammatory responses. FcR belong to the diverse multigene Ig superfamily that either activates or inhibits cellular responses (Ravetch, 1994; Daeron, 1997). Like T-cell receptor (TCR) and BCR, activating FcR are members of the immunoreceptor tyrosine-based activation motif (ITAM) family, which is composed of a ligand-binding α chain and an associated common γ chain (Reth, 1989; Kurosaki and Ravetch, 1989; Cambier, 1995). Therefore, mice with a targeted disruption of the γ chain fail to express the high-affinity IgE receptor, FcεRI, the high-affinity IgG receptor FcγRI, and the low-affinity activation receptor, FcγRIII (Miyajima *et al.,* 1997). Analysis of these mice revealed a major role of IgG in evoking an anaphylactic response when bound to FcγRIII on effector cells, including mast cells, neutrophils, macrophages, and natural killer (NK) cells.

The role of IgE and its receptors in the clearance of helminth infections and the role of IgG FcR in immunity to microbial pathogens such as streptococcus have been addressed by several studies. The results suggest that susceptibility to infection by these pathogens is not influenced by the presence or absence of FcR genes, as no differences are observed between knockout mice and their heterozygous littermates (summarized in Ravetch and Clynes, 1998). Similarly, analysis of different FcR$^{-/-}$ mice during VSV or LCMV infections also did not reveal a role for FcR in control of these viruses. Neutralizing antibody responses, virus-specific cytotoxic T cell responses, and viral clearance in infected hosts were similar in FcR$^{-/-}$ mice and heterozygous littermates (M. Pericin, unpublished results).

Taken together, although FcRs may play a role in anaphylactic reactions, antibody-mediated autoimmune diseases, and probably even some tumor model situations, so far there is no evidence that FcR are crucially involved in protection against a variety of bacterial, fungal, protozoan, helminth, or viral pathogens (Ravetch and Clynes, 1998).

V. The Role of Antigen Structure, Organization, and Dose on B Cell Responses

The neutralizing antibody response against VSV, poliovirus, and some other viruses is induced very rapidly; an IgM response is measurable by day 2, peaks by days 4 to 6, and is largely T-cell-independent during this early phase. Nude or CD4 T-cell-depleted mice mount a good IgM response if intact wild-type virions (live, UV-, or formalin-inactivated) are used for immunization. Thus, the highly organized, repetitive, densely packed, neutralizing epitopes that are usually

exhibited on the tip of glycoproteins of the virus envelope are able to trigger strong IgM responses in the absence of T help (T-help-independent type 1, TI-1). Moreover, these epitopes are often the only ones accessible to nAbs or B cell receptors (BCRs). This was first demonstrated for influenza viruses where it was shown that antibodies cannot squeeze in between the hemagglutinin epitopes on intact virions (Lafferty, 1963; Fazekas de St. Groth, 1981). The same has been shown for VSV-neutralizing epitopes expressed by the single-envelope glycoprotein (Lefrancois and Lyles, 1983; Lefrancois, 1984; Wagner, 1987; Luo *et al.,* 1988; Roost *et al.,* 1995, 1996). In contrast, low doses of monomeric glycoprotein or hemagglutinin carrying the neutralizing determinants will not induce this early and efficient IgM response (Bachmann *et al.,* 1997a). Of course, these early antibody responses are dependent on viral dose and probably on natural antibodies and complement factors binding to complexes, as discussed above (Ochsenbein *et al.,* 2000a). Neutralizing epitopes expressed on infected cells exhibit a less repetitive and less rigid distribution. Such cell surface multimers represent a third form of neutralizing viral epitopes and induce yet a different T-cell-dependent IgM response. In this case, the necessary T help does not have to be linked in the conventional manner and noncognate bystander T help is sufficient to help this IgM response (TI-2) (Bachmann *et al.,* 1993; Bachmann and Zinkernagel, 1997). These results indicate that the rigid paracrystalline form of the glycoprotein tips, spaced 8 to 10 nm, cross-link specific BCRs very efficiently. This process requires at least 10 to 30 determinants to trigger B cell differentiation, activation and antibody production (Dintzis *et al.,* 1976, 1989; Bachmann *et al.,* 1993; Bachmann and Zinkernagel, 1996, 1997; Fehr *et al.,* 1996, 1998a). Although efficient cross-linking of BCRs enhances B cell activation it should be noted that both TI-1 and TI-2 type IgM responses are also dose dependent, since low doses are completely T dependent (Freer *et al.,* 1994; Ochsenbein *et al.,* 2000a). However, with sufficient doses the IgM response is T independent and very rapid for both TI-1 and TI-2. This suggests that the distinction between TI-1 and TI-2 is probably not of great importance from a pathophysiological point of view. However, the distinction may be relevant for self-/non-self-discrimination and autoimmunity (Bachmann and Zinkernagel, 1997). The above findings support the recent model proposing that BCRs form clusters of about 10 to 20 receptors and that this clustering may be essential for B cell selection and induction (Reth *et al.,* 2000). Because antigen-mediated cross-linking of a minimal number of BCRs is necessary and sufficient to induce B cell proliferation and antibody production, it indicates that antigen signals in the absence of so-called second signals do not anergize or delete B cells, as postulated by others (Bretscher and Cohn, 1970; Mueller *et al.,* 1989; Schwartz, 1989; Cohn and Langman, 1990; Langman and Cohn, 1993; Matzinger, 1994).

B cells seem to take antigen organization as a marker for reactivity (Bachmann *et al.,* 1993, 1995; Bachmann and Zinkernagel, 1996, 1997; Ochsenbein and

Zinkernagel, 2000) Indeed, accumulating evidence suggests that the more regular and rigidly ordered the identical antigenic determinants are, the less the B cells are dependent on and controlled by T cells. These multimeric paracrystalline structures are the hallmark of surface antigens on infectious agents and therefore must always be considered as non-self. This has obvious consequences for our understanding of antibody-mediated autoimmunity but also offers a new concept in self-/non-self-discrimination by B cells. Again, it needs to be emphasized that the dose and persistence of antigen are as important as antigen structure. However, the relative importance of these two factors cannot be defined because they cannot be separated experimentally; i.e., multimeric antigens are bigger than monomeric antigens which therefore affects both dose and structure (Dintzis *et al.,* 1989; Ochsenbein and Zinkernagel, 2000). As will be pointed out later, similar structures may exist in self-antigens, but they are usually not accessible to B cells in healthy individuals. In contrast to multimeric non-self antigens, monomeric determinants are the hallmark of self-antigens in blood and the lymphatic system. B cell responses to self-antigens are therefore strictly regulated by cognate T help which is absent for self-antigens (Weigle, 1973). Efficient cross-linking of BCRs is required in order to rapidly expand B cells during the early phase of an infection and provide sufficient IgM titers to limit systemic virus distribution. Subsequently, when virus-specific T helper cells are induced around day 4 to 6 post infection they can now encounter this expanded B cell pool and may efficiently induce the switch to IgG (Roost *et al.,* 1990; Bachmann and Zinkernagel, 1996, 1997; Baumgarth, 2000). This IgG response is longer lived (20-d half-life) and more protective than the IgM response (1- to 3-d half-life). IgG molecules also diffuse more efficiently into solid tissues and the central nervous system as they are smaller than IgM molecules. Nevertheless, IgM can very efficiently prevent hematogenic spread of virus, as has been suggested by the survival of VSV-infected $CD40^{-/-}$ or CD4-depleted mice which are incapable of switching to IgG (Oxenius *et al.,* 1996). Rapid amplification of rare B cells can have both beneficial and detrimental consequences for the host. On the one hand, an efficient neutralizing or protective antibody response against acute cytopathic infections is generated, while on the other hand auto-antibodies and autoimmune disease could potentially be induced.

The frequency of neutralizing antibody-producing B cells is on the order of one per 10^5–10^6 spleen cells, corresponding to about one per 10^4–10^5 B cells (Bachmann *et al.,* 1994b). This frequency is considerably lower than frequencies determined for hapten-specific dinitrophenyl (DNP)- or trinitrophenyl (TNP)-specific B cells that are on the order of 10^{-2} (Bos and Meeuwsen, 1989). Similarly frequencies for complex protein antigens such as lysozyme, ovalbumin, or bovine serum albumin are about 10–100 times higher than for neutralizing epitopes, a fact that correlates with the many more antigenic determinants that can be recognized by B cells on these complex globular proteins compared to the

usually unique neutralizing epitope exposed on intact viral surfaces (Roost *et al.,* 1995, 1996; Bachmann and Zinkernagel, 1997; Goldbaum *et al.,* 1999). Thus, neutralizing antiviral antibody responses protect efficiently against viral infections, whereas antibody responses against the many internal viral antigens or peptides from the surface glycoproteins are usually not protective. The reason is simply due to the structure of the limited size of the exposed determinant accessible to antibodies. In many cases, the neutralizing epitope is the sole determinant regularly accessible to B cells in the intact viral surface. Alternatively, it is the most peripheral of the available determinants (e.g., poliovirus), and antibodies may therefore efficiently prevent docking to the appropriate receptor. Neutralizing epitopes on many (if not most) viruses, therefore, have been selected by long-time co-evolution to represent single antigenic sites critical for prevention of virus infection. It is, therefore, not a surprise that very closely related viruses share greater than 95% of proteins and sequences of internal structural and nonstructural antigens but vary in single neutralizing antigenic sites forming the neutralizing epitope; these differences define what we call *serotypes*. By definition, serotypically distinct viruses induce no cross-protection by the respective nAbs, despite the fact that they share most if not all T helper cell and cytotoxic T cell determinants (Gupta *et al.,* 1986; Roost *et al.,* 1990; Zinkernagel, 2000b). It also cannot be overemphasized that antibodies against internal viral antigens in general are irrelevant and of no importance for antiviral protection.

The structure of neutralizing determinants is usually defined by an area of about 500 to 1000 A^2 built from several protein loops. For rotaviruses, influenza, and arenaviruses three loops seem to be involved in forming neutralizing determinants; the situation is probably in reality even more complex because the relevant glycoproteins are composed of three to four interacting units (Webster *et al.,* 1982; Jackson *et al.,* 1982; Burns and Buchmeier, 1993). In a few selected cases, linear epitopes have been shown to induce nAbs—for example, against foot-and-mouth disease virus (Bittle *et al.,* 1982; Brown, 1988) or gp41 (Xiao *et al.,* 2000) and V3 loop of HIV (Robinson and Mitchell, 1990; Poignard *et al.,* 1996). But, in general these exceptions only confirm the rule that neutralizing sites are formed by complex loop structures. The V3 loop of HIV may be a particularly revealing example, as antibodies induced by this loop only neutralize artificial lab strains and usually do not confer protection from infection with primary isolates. In fact, the neutralizing epitope on the HIV glycoprotein seems to be extremely conformation dependent and composed of at least two separate regions of the protein engaged sequentially during infection (Cho *et al.,* 2000). Neutralizing epitopes have been determined by X-ray crystallography for poliovirus, influenza, adenoviruses, and rhinoviruses. For many viruses, this information is still missing, but some approximations have been deduced from mutational analysis. However, the indirect relationship

between amino acid exchanges and structural consequences renders interpretations of these results generally difficult (Laver *et al.,* 1990). As far as analyzed, the simple notion that nAbs cover the unique antigenic sites exposed on the virus surface and/or bind to the receptor-binding structures may still be an oversimplification but remains an attractive and perhaps rather general explanation (see later).

VI. T-Cell Dependent Activation of B Cells in Viral Infections

General rules of T–B cooperation and induction of T helper cells by macrophages, antigen-presenting cells (APCs), or virus-specific B cells are as described in model situations in textbooks (Paul, 1993). Nevertheless, some very interesting observations can be made by analyzing T–B cell interactions during protective neutralizing antibody responses. They involve, first, the limiting frequencies of neutralizing B cells and T helper cells and second, the relative importance of T help specific for viral envelope versus internal antigens.

As pointed out above, neutralizing B cells are of very low frequency. Therefore, induction of these rare B cells must be very efficient, particularly during the early phase of a viral infection when antigen is scarce and usually strictly localized. Not surprisingly, B cells are the major limiting factor during neutralizing antibody responses, not the amount nor the activation state of T helper cells (Charan and Zinkernagel, 1986). This was documented first by immunization of mice with one serotype of VSV (VSV-IND) and after 3 months' challenging with a second VSV serotype (VSV-NJ). The two VSV serotypes share virtually all helper and cytotoxic T cell epitopes; therefore, the first infection should prime all specific CTL and T helper cells. The challenge infection revealed, however, that the neutralizing antibody response generated was strictly of a primary type (Charan and Zinkernagel, 1986; Gupta *et al.,* 1986). This finding suggests that priming of B cells rather than of helper T cells may be of importance for inducing protective immunity mediated by antibodies. This contrasts with the conventional view developed from model studies analyzing specific B cell responses in classical hapten carrier systems (Mitchison, 1971; Katz and Benacerraf, 1972). These early studies clearly showed that hapten-specific B cells were not limiting, whereas the amount of available and primed T help did limit the antibody response both qualitatively and quantitatively. However as pointed out above, the frequency of hapten-specific B cells is usually very high, on the order one per 10^2 to 10^3, contrasting with the much lower frequency of virus-specific neutralizing B cells (one per 10^4 to 10^5). In summary, these experiments provide strong evidence that B cells are the limiting factor in neutralizing antibody responses against many acute cytopathic viruses and presumably also against bacterial toxins or other proteins on bacteria and parasites (Charan and Zinkernagel, 1986; Roost *et al.,* 1990; Bachmann and Zinkernagel, 1997; Zinkernagel, 2000b). This correlates well with

clinical observations that patients having survived an infection with poliovirus 1 or influenza HA1 are not protected against a subsequent infection with a different viral serotype (Yewdell *et al.,* 1979; Nathanson and Martin, 1979; Sabin, 1981, 1985; Fazekas de St. Groth, 1981; Webster and Rott, 1987; Laver *et al.,* 1990; Liang *et al.,* 1994; Nathanson and McFadden, 1997; Ada, 2000). The crucial role of neutralizing antibodies in antiviral protection does not exclude the possibility that highly activated effector cytotoxic T cells or T helper cells could also participate in protection (Zweerink *et al.,* 1977; Effros *et al.,* 1979). However, because antigen is eliminated quickly, this T cell effector phase is usually short (2 to 3 weeks) (Roost *et al.,* 1990) and importantly cannot be easily induced and maintained by vaccines, as will be discussed below.

T cells specific for either envelope glycoproteins or any of the internal antigens are equally efficient in their ability to help neutralizing B cells switch from IgM to IgG (Liang *et al.,* 1994; Oxenius *et al.,* 1998b). Interestingly, however, the switch of B cells specific for internal antigens is virtually exclusively mediated by T helper cells specific for that particular antigen. In contrast, T cells specific for any viral antigen can support switching of B cells specific for viral surface proteins. These observations strongly support the view that intact virus particles are taken up by neutralizing antibody-producing B cells, which will subsequently present any of the viral antigens on major histocompatibility complex (MHC) class II, whereas the switch of B cells specific for internal viral antigens requires that such antigens be released from infected cells or viral particles in order to be taken up.

VII. B Cell Unresponsiveness

Historically, negative selection of B cells expressing specificities for self-antigens has been postulated as a major mechanism for maintenance of tolerance. Both Ehrlich's horror autotoxicus (Ehrlich, 1906) and Burnet's proposal of negative selection of immune reactivity, at the time when the distinction between T and B cells was not yet known, focused exclusively on B cells (Burnet and Fenner, 1949). These notions were expanded by Nossal and co- workers to include the concept of anergy (Nossal, 1983). More recently, IgM–BCR transgenic models have been used to document negative selection as the basis for the absence of auto-antibody responses (Goodnow *et al.,* 1989; Nemazee and Buerki, 1989; Nemazee and Buerki, 1989; Nemazee *et al.,* 1991). Lysozyme-specific IgM transgenic mice crossed to mice expressing lysozyme showed that the number of specific B cells was reduced compared to controls (Goodnow *et al.,* 1989). Similarly, anti-H-2K^k IgM transgenic B cells were largely deleted when these transgenic mice were crossed to H-2K^k but not to H-2^d mice (Nemazee and Buerki, 1989). Since the same antigen expressed as a soluble product in

serum failed to delete the B cells to a comparable extent, the conclusion in both transgenic IgM situations was that membrane-bound self-antigens delete self-specific B cells efficiently, whereas soluble antigens in serum fail to do so (Nemazee *et al.,* 1991; Basten *et al.,* 1991). The conceptual views based on these experiments are in conflict with a number of clinical and other experimental observations that do not completely support negative selection or anergy models. In fact, auto-antibodies can readily be detected and have often been observed during infections, particularly chronic infections, not only against soluble self antigen but also against membrane-expressed antigens (e.g., acetylcholin receptor). The recognition that polyclonal stimulators such as lipopolysaccharides could stimulate B cells in the absence of cognate T help revealed the presence of B cells that readily secreted auto-antibodies in normal individuals (Coutinho and Moller, 1975; Moller, 1975). One inherent problem of these observations and their interpretation is that the assays measuring auto-antibodies or autoreactive T cells do not necessarily reflect *in vivo* reactivity. Nevertheless, most clinically relevant autoimmune diseases involve one or more auto-antibody specificities (Teale and Mackay, 1979; Rose and Mackay, 1992). This by itself is a strong indication that auto-antibodies and autoreactive B cells are not a rare exception.

A. B Cell Unresponsiveness Studied with Viruses

The data obtained from antibody transgenic models differed from those observed in a separate study of antibody responses against a VSV-neutralizing epitope expressed in mice as a membrane-associated or soluble antigen. In both mice, immunization with intact virions (live, UV-irradiated, or formalin-fixed) promptly induced a T-cell-independent IgM response (Zinkernagel *et al.,* 1990; Bachmann *et al.,* 1993). Since intact virions contain internal antigens offering T help, these neutralizing antibody responses were efficiently switched to IgG. In contrast, purified VSV-G delivered in adjuvant failed to induce neutralizing IgM or IgG responses. These results indicated that high-affinity B cells were still present in these VSV-G transgenic mice. In addition, they suggested that B cells may be less responsive to monomeric and oligomeric antigens, whereas they would be triggered very efficiently by highly repetitive self antigens. More recently, these findings were confirmed in anti-K^k transgenic mice that responded to a mimicking antigen coupled to bacterial viruses (Kouskoff *et al.,* 2000). It remains to be evaluated whether these results parallel the findings obtained with VSV or reflect the presence of very low avidity antibodies assessed by ELISA.

Another example that demonstrates that B cells are, in general, not tolerant or anergic, even to high levels of soluble antigen, is seen in CD8-deficient mice when infected with the noncytopathic LCMV. These mice cannot eliminate virus after acute infection and become carriers with high viral titers in most organs and 10^5 to 10^6 pfu/ml of serum. Such carrier mice possess antibodies, as detected by ELISA, specific for several viral antigens (Hotchin and Sikora, 1964; Oldstone

and Dixon, 1967; Oldstone *et al.,* 1980; Moskophidis *et al.,* 1987). Interestingly, 40 to 50 days after infection of $CD8^+$ T cell-deficient mice, high titers of antiviral nAbs are generated which are sufficient to eventually eliminate the virus even in the complete absence of cytotoxic T cells (Ciurea *et al.,* 2000). Thus, also in this model, B cells are not tolerant and the T helper cells induced early after infection are sufficient to provide the necessary T help. This T help may eventually be exhausted if virus persists at high levels. One could still argue that even high virus titers in the blood and solid organs are not sufficient to tolerize B cells. However, mice infected *in utero* by carrier mothers become tolerant at helper and cytotoxic T cell levels and are unable to generate nAbs (Rowe, 1954; Hotchin, 1962; Lehmann-Grube, 1971), suggesting that, in this case, availability of T help is the limiting factor rather than potentially autoreactive B cells (Battegay *et al.,* 1994; Matloubian *et al.,* 1994; Oxenius *et al.,* 1998a,b; Seiler *et al.,* 1999).

B. General Characteristics of Autoimmune B Cell Induction

Accumulating evidence suggests that B cells are not negatively selected. Instead, either they fail to be induced by low doses of monomeric antigen in the absence of T help or they only generate short-lived IgM responses that have little pathological consequences for the host (Ochsenbein *et al.,* 2000a). Efficient antigenic cross-linking of the BCR together with the binding of complement to marginal zone macrophages are key to triggering an efficient IgM response that will switch to a long-lasting IgG response only in the presence of classical cognate T help (Odermatt *et al.,* 1991; Seiler *et al.,* 1997; Ochsenbein *et al.,* 1999b). It is via these parameters—antigen structure, antigen dose, time duration, and availability of T help—that long-lasting antibody responses are regulated.

What can we learn from antiviral antibody responses for the understanding of how auto-antibody responses are generated? If it is true that antigen structure, antigen amount, and availability of T help are the critical factors in regulating the usual absence of B cell and auto-antibody responses in a young host, then the induction of these responses should reflect a breakdown or defect in one or more of the regulatory requirements. From a basic point of view, it is interesting to note that auto-antibody responses and autoimmune diseases often comprise specificities for highly repetitive determinants such as collagen, DNA, acetylcholine receptors, etc. (Dintzis *et al.,* 1976, 1989; Zinkernagel *et al.,* 1991; Bachmann *et al.,* 1993; Bachmann and Zinkernagel, 1996, 1997; Schulte *et al.,* 1998). This observation supports the idea that cross-linking by polymeric determinants triggers B cells very efficiently, thus amplifying their frequency so it is more likely that they could be switched by either specific or bystander T help.

It is worth emphasizing here that the initiation of many auto-antibody responses and diseases are associated with infections (Vaughn *et al.,* 1989; Rose and Mackay, 1992; Jansen *et al.,* 1993; Ludewig *et al.,* 1998, 1999). Presumably, infections can lead to the release of large amounts of self-antigen, generate

inflammatory signals, and activate antigen-presenting cells. Also it is particularly interesting to note that many chronic auto-antibody immune responses are associated with the generation of new secondary lymphoid structures in the target organ for which the auto-antibodies are specific. Haschimoto's thyroiditis, Sjögren's disease, and rheumatoid arthritis are excellent demonstrations of where the formation of new secondary lymphoid tissue correlates with chronic persistence of auto-antibody responses. Such neo-formation can also be induced by repetitive injection of dendritic cells presenting a neo-self-antigen (Ludewig *et al.,* 1999). Chronic infections that persist in the periphery may also trigger formation of lymphoid structures and enhance autoimmune B cell responses. In summary, induction and maintenance of auto-antibody responses can be enhanced by repetitive auto-antigen structures and by the chronicity of the response leading to the perpetuation of inflammatory events and formation of new lymphoid tissue within target organs.

To further investigate the potential of repetitive antigen structures in initiating auto-antibody responses, virus particles were used as matrix to display antibodies in a highly repetitive fashion, and the generation of anti-antibodies, similar to that found in rheumatoid arthritis, was monitored (Fehr *et al.,* 1997). Mice immunized with either VSV particles or a monoclonal-neutralizing IgM antibody did not develop any anti-antibodies, whereas mice immunized with VSV particles complexed with anti-VSV antibodies readily mounted anti-idiotype-specific antibody responses. Similarly, when gram-negative bacteria complexed with monoclonal antibodies were used for immunization, rheumatoid factors (antibodies against constant antibody regions) were induced but not when the antibody or bacterium were injected alone. In this situation, injection of lipopolysaccharide (LPS) together with the complex was necessary in order to break B cell unresponsiveness to a self-antigen normally present in high concentration in serum. This study illustrates the potential for high concentrations of repetitive antigen structures to induce auto-antibodies. At least two other examples of auto-antibody induction using a similar approach have been reported. Recombinant bovine papillomaviruses were constructed to express a peptide of the extracellular loop of the mouse chemokine receptor 5 (CCR5) within the immunodominant epitope of the main papillomavirus envelope protein L1 (Chackerian *et al.,* 1999). Injection of this recombinant papillomavirus induced anti-CCR5 auto-antibodies which blocked regulated upon activation of normal T cell expressed and secreted (RANTES) binding and cellular entry of HIV, although this occurred without any apparent induction of autoimmune disease. However, induction of clinical autoimmunity has been described in patients with hepatitis C virus (HCV) infection. In perfect analogy to the anti-antibody study described above, serum of patients suffering from chronic HCV infection contains immune complexes of HCV–anti-HCV antibodies and cryoglobulins (a distinct type of rheumatoid factor). Interestingly, electronmicroscopic analysis revealed that these complexes have highly repetitive paracrystallin structures (Szymanski

et al., 1994). Deposition of these complexes in tissues such as kidney or skin can lead to the autoimmune disease known as mixed cryoglobulinemia.

VIII. Examples of Nonclassical Virus Neutralization

Before going into some special but revealing examples of neutralizing antibody responses against viruses, let us restate some general rules (Fenner, 1949; Sabin, 1981; Mims, 1987; Nathanson and McFadden, 1997; Zinkernagel, 2000b). Following virus infection of the mucosa, local IgA immune responses are probably important but are still poorly understood (Brandtzaeg, 1989; Macpherson *et al.,* 2000). Viral spreading to Peyer's patches or draining lymph nodes will induce a primary immune response of both B and helper T cells, resulting in local and systemic IgA, IgM, and IgG production. Subsequent to local infection, the virus will usually replicate in draining lymph nodes for 1 to 3 days and then spread systemically (Johnson and Mims, 1968; Mims, 1987). This leads to distribution of antigen in target organs, including skin, central nervous system, kidney, lung, and spleen. Systemic distribution is important to enable the virus to spread horizontally via aerosols, stool, or urine, but the spreading of cytopathic viruses may kill the host if not controlled early by IgM. The immune response initiated in local lymph nodes and spleen will eventually control both systemic and local infection. The sequential induction of immune responses is of key importance to control generalized infections (Fenner, 1949), although entrapment of virus within secondary lymphoid organs may also be important, particularly to rapidly induce responses against viruses that reach the blood directly, such as arthropod-borne viruses.

A. Virus Stays Outside of the Immune System

Papillomaviruses exclusively infect skin or mucosal cells, usually basal cells of the epithelium, in a strictly localized fashion and they replicate productively only in terminally differentiated cells (e.g., keratinocytes) (Tindle and Frazer, 1994; Frazer *et al.,* 1999). Effector T and B cells and even Langerhans cells cannot easily access these cells in the skin. In addition, differentiated cells do not normally release antigen or emigrate to local lymph nodes. Therefore, papillomavirus antigens are initially not transported to the lymph nodes and such infections may be ignored by the immune system for long periods of time (Kundig *et al.,* 1995; Ochsenbein *et al.,* 2000b).

Rabies virus uses comparable tactics, but, rather than skin, it initially resides within axons. During retro-axonal flux, the virus is not accessible to T or B cells and does not reach secondary lymphoid organs. At this stage of infection rabies virus is not yet cytopathic; therefore, viral antigen will not be picked up by macrophages or other APC and thus will not reach draining lymph nodes. Once the virus has reached the neuronal body and lyses it, virus spread appears to be so rapid that the immune response is usually induced too late to be of benefit

for the host. Post-exposure vaccination with inactivated virus seems to shorten this delay and may deliver virus antigen to draining lymph nodes. This induces nAbs that may, under favorable timing conditions, limit ongoing virus transport and prevent substantial central nervous tissue damage (Baer and Cleary, 1972; Murphy, 1977).

B. Antibody-Dependent Enhancement of Infection

Normally, antibodies provide protection against viruses but in some cases they may actually enhance infection. Enhancement of viral infectivity has been mostly studied *in vitro* with cells susceptible to virus infections and bearing Fc and/or complement receptors. Antibody-coated virus can infect cells via FcR rather than via a specific receptor. Many non- or low-cytopathic viruses are suspected to use such antibody enhancement for infection, including HIV (Takeda *et al.,* 1988; Robinson *et al.,* 1988; Homsy *et al.,* 1989), Dengue viruses (Halstead, 1988), and perhaps HCV. The first observations made with flaviviruses by Hawkes *et al.* (Hawkes, 1964) were largely extended by Halstead and coworkers (Halstead, 1988). They describe an antibody-dependant enhancement of Dengue virus replication of about 100-fold in cultures of peripheral blood leukocytes of humans or primates. To what extent serotype differences contribute to viral infection via FcR and whether or not antibody-dependent enhancement plays a major role *in vivo* is, however, still not very clear for viruses other than Dengue. However, low-avidity antibodies may enhance early uptake of a new serotype and therefore render a second infection in some immune patients more pathogenic than the first infection (Burke *et al.,* 1988; Morens, 1994; Thein *et al.,* 1997). This is the classical explanation for severe hemorrhagic disease after repetitive Dengue virus infections, but similar mechanisms may apply to persisting viruses that escape nAbs. It may also explain the early death of kittens inoculated with infectious feline peritonitis virus following passive transfer of antiviral antibodies (Weiss and Scott, 1981). In addition, arenavirus infections including LCMV have shown enhancement *in vitro* and likely also *in vivo* (Lewis *et al.,* 1988; Ochsenbein *et al*., 1999a).

C. Immunosuppression

Acquired immunosuppression by virus-specific $CD8^+$ lymphocytes occurs when these cells destroy antigen-presenting cells, such as dendritic cells or macrophages, and thereby impair induction of immune responses (Mims and Wainwright, 1968; Bro-Jorgensen and Volkert, 1974; Silberman *et al.,* 1978; Biberfeld *et al.,* 1985; Racz *et al.,* 1986; Leist *et al.,* 1988; Odermatt *et al.,* 1991; Althage *et al.,* 1992; Tishon *et al.,* 1993; Borrow *et al.,* 1995; Seiler *et al.,* 1997). Such a mechanism might help to explain the long delay in the appearance of neutralizing antibodies following noncytopathic virus infections such as HIV (Moore *et al.,* 1994; Pilgrim *et al.,* 1997; Carotenuto *et al.,* 1998), HBV (Alberti

et al., 1988) HCV in humans, or LCMV in mice (Planz *et al.,* 1996). Detailed analyses of this process during LCMV infection has revealed the following interesting virus–host immune relationship (Zinkernagel *et al.,* 1999). During the early phase of infection, LCMV seems to preferentially infect splenic marginal zone macrophages (Mims and Tosolini, 1969; Jacobs and Cole, 1976) and dendritic cells in the periphery and secondary lymphoid organs. These infected cells appear to be subsequently destroyed by early induced effector cytotoxic T cells (Odermatt *et al.,* 1991; Althage *et al.,* 1992; Tishon *et al.,* 1993; Borrow *et al.,* 1995; Sevilla *et al.,* 2000). Treatment with CD8-depleting antibodies prevents acute immunopathology in lymphohematopoietic organs (Odermatt *et al.,* 1991; Battegay *et al.,* 1993b; Binder *et al.,* 1997). The immunopathological destruction of antigen-presenting cells of the marginal zone hampers both humoral and T-cell-mediated immune responses (Odermatt *et al.,* 1991; Seiler *et al.,* 1997). This virus-induced acquired immunosuppression seems to facilitate the establishment of long-term persistence of the infecting noncytopathic virus. For LCMV it has been shown that antibody responses against the internal viral antigens, including nucleoprotein or the membrane-anchored part of the glycoprotein, are not drastically impaired by this generalized immunosuppression (Battegay *et al.,* 1993b). However, the neutralizing antibody response is greatly reduced or even prevented completely for 70 to 150 days. This difference correlates with the observation that hybridomas specific for neutralizing determinants were frequently infected with LCMV, whereas those specific for the nucleoprotein were not (Planz *et al.,* 1996). These findings suggest that intact virus particles were taken up by neutralizing antibody-specific B cells via the specific BCR and that virus could replicate within these cells. Infected B cells, therefore, express viral peptides in association with MHC class I antigens and become susceptible to destruction by cytotoxic T lymphocytes (CTLs). In contrast, nucleoprotein-specific B cells are not productively infected, will not express peptides on MHC class I, and are therefore not susceptible to CTL lysis (Planz *et al.,* 1996). The parallels between the kinetics of neutralizing antibody responses of human noncytopathic or poorly cytopathic viral infections (HBV, HCV, and HIV) and LCMV are striking. In all these infections, neutralizing antibody responses become detectable only when, or a few months after, virus titers have been reduced to very low or undetectable levels.

D. Sensitivity to Antibody Neutralization Versus Tropism

As both viral sensitivity to antibody neutralization and viral tropism are determined by structures on surface envelope glycoproteins, modifications of cell tropism may alter the neutralization epitopes and vice versa. These interactions are best illustrated during murine infection with lactate dehydrogenase-elevating virus (LDV), an arterivirus which causes asymptomatic persistent infections through cytopathic replication in a renewable subpopulation of macrophages

(Plagemann *et al.,* 1995). Persistence is favored by specific polylactosaminoglycan chains on the ectodomain of the primary envelope glycoprotein which masks the single antibody neutralization epitope and therefore decreases viral immunogenicity (Chen *et al.,* 2000). LDV strains that lack these specific chains are sensitive to antibody neutralization and are not able to establish lifelong infections. However, these strains have also been shown to be neuropathogenic, as the missing glycosylation allows viral interaction with a putative receptor on anterior horn neurons, causing a paralytic disease in conditions of impaired neutralizing antibody responses.

A similar interaction has been suspected for HIV, where macrophage or T cell tropism is determined by the envelope glycoproteins binding to CD4 and co-receptors. As nAbs have been shown to be able to prevent the CD4-dependent association of gp120 with CCR5 (Trkola *et al.,* 1996), changes in neutralization sensitivity could directly affect HIV tropism. Subsequent studies, however, have demonstrated that antibody-mediated neutralization of HIV is independent of co-receptor usage (Trkola *et al.,* 1998; Montefiori *et al.,* 1998; LaCasse *et al.,* 1998).

IX. Virus Escape from Neutralizing Antibody Responses

The molecular basis for viral immune selection lies in the extensive genetic variation of viruses generated by mutation, recombination, or reassortment of genomic segments. RNA viruses have a particularly high spontaneous mutation rate (10^{-3} to 10^{-5} substitutions per nucleotide per round of replication). This is a consequence of absence of proofreading-repair mechanisms of RNA polymerases and retroviral reverse transcriptases (Holland *et al.,* 1982). These viruses are therefore not present as a homogenous population in their host, but circulate as quasi-species. The evolution of quasi-species depends on the population size of the virus, the competitive fitness of variants during their life cycle, and environmental factors (immune responses, particular cells or tissues in the host, drugs) acting through continuous positive selection pressure on viral recognition and tropism (Domingo and Holland, 1997).

Within quasi-species, virus variants containing mutations in envelope glycoproteins that could alter recognition by nAbs would be positively selected in situations where the humoral response plays a substantial role for virus control. Several mechanisms may account for the immune escape of neutralization-resistant mutants. First, amino acid substitutions within the neutralization determinant may alter the affinity of nAb for the virion (Wiley *et al.,* 1981). Second, mutations at distant sites may change the global conformation of the antigenic determinant (Diamond *et al.,* 1985). Finally, mutations may allow additional glycosylation sites which may mask neutralizing epitopes (Skehel *et al.,* 1984; Wright *et al.,* 1989; Reitter *et al.,* 1998; Kimata *et al.,* 1999; Chen *et al.,* 2000).

A. Influenza Viruses

Antigenic variation in viruses of the Orthomyxoviridae family, which are composed of a segmented RNA genome, have long been a paradigm for the study of escape from nAb responses (Webster *et al.,* 1982; Wilson and Cox, 2000). First, influenza type A viruses may show genetic reassortment between human and animal strains, mainly from avian and swine reservoirs. Thus, novel pandemic strains emerge which have distinct antigenic characteristics in their surface glycoproteins, hemagglutinin (HA) or neuraminidase (NA) (Cox and Subbarao, 2000). This phenomenon is known as *antigenic shift*. Second, accumulation of single-point mutations within the HA and NA genes of influenza A and B viruses can occur naturally. This process, called *antigenic drift*, is responsible for the annual recurrence of influenza epidemics. The amino acid changes are clustered within five, but mainly in one to three, of the major antigenic sites on the surface glycoproteins (Wiley *et al.,* 1981). Several observations indicate that these mutations are selected by an ongoing nAb response. Variants with similar mutations have been selected with monoclonal antibodies (Yewdell *et al.,* 1979). Furthermore, there is evidence of variation in the antibody repertoire of individual mice immunized with influenza (Staudt and Gerhard, 1983). Finally, neutralization escape mutants were readily selected by sera from immune mice (Lambkin *et al.,* 1994), indicating a restricted antibody repertoire in each animal. Although individuals are rarely able to induce an antibody response against all five neutralizing domains, a whole spectrum of anti-HA responses can be found in the human population (Wang *et al.,* 1986). Therefore, epidemiologically significant antigenic variants may arise only through selection at the population level after accumulation of sequential mutations. In line with these observations, it has been shown that four or more amino acid changes occurring in at least two of the five antigenic sites of HA are needed for variants during antigenic drift to be of epidemiological relevance (Wiley *et al.,* 1981).

Although antigenic variants of other cytopathic RNA viruses (rabies virus, poliovirus, foot-and-mouth disease virus, measles virus) have been selected in the presence of neutralizing monoclonal antibodies (Diamond *et al.,* 1985; Mateu *et al.,* 1989; Schrag *et al.,* 1999; Borrego *et al.,* 2000), the *in vivo* role of escape from humoral responses for viral persistence during natural infections is unclear (Hovi *et al.,* 1986) and may not be of importance (Gebauer *et al.,* 1988). An alternative explanation for antigenic diversification may be that there is random occurrence of tolerated amino acid replacements within antigenic sites which may have less stringent structural requirements because they are located on the surface of envelope glycoproteins (Domingo *et al.,* 1993). Furthermore, amino acid substitutions may occur as a consequence of other selective forces, such as changes in virus–cell receptor interactions and tropism, which would

only secondarily affect antigenic specificity (Both *et al.,* 1983; Kaplan *et al.,* 1990).

B. Hepatitis B Virus

Although hepatitis B virus (HBV) has a double-stranded DNA genome, its life cycle includes an intracellular RNA pregenomic intermediate which is reverse-transcribed within the nucleocapsid (Lee, 1997). This step is responsible for the high viral mutation rate found with HBV. In association with cellular immune responses, nAbs to HBV envelope antigens play a major role in viral clearance as they are readily detectable in patients who clear the virus but not in patients with chronic HBV infection (Chisari and Ferrari, 1995). Antigenic variation in surface glycoproteins has been shown to be clinically important from several points of view. First, vaccine-induced escape mutants of HBV have been described (Carman *et al.,* 1990) showing an incidence of 2 to 3% in endemic regions. Second, passive immunoprophylaxis with polyclonal HBV-specific Igs in order to prevent perinatal or transplantation-associated viral transmission may select nAb-escape variants (Carman *et al.,* 1996; Hsu *et al.,* 1997; Protzer-Knolle *et al.,* 1998). Third, naturally occurring mutations in genes coding for surface proteins were associated with viral persistence (Ogura *et al.,* 1999). Finally, detection of HBV in patients infected with virus containing mutations within the surface antigen (HbsAg) may not be possible using standard HBsAg assays (Carman *et al.,* 1995), a problem which is of great public health significance.

C. Hepatitis C Virus

Hepatitis C virus (HCV) is a positive-sense single-stranded RNA virus of the Flaviviridae family (Rice, 1996). Several lines of evidence indicate that the nAb response exerts selection pressure on the hypervariable region 1 (HVR1) of the E2 envelope glycoprotein. Emergence of autologous nAb–escape HVR1 variants have been observed during chronic HCV infection (Weiner *et al.,* 1992; Shimizu *et al.,* 1994). In an experimental HCV infection model of chimpanzees, anti-HVR1 antiserum induced protection against homologous HCV infection but not against the emergence of neutralization escape mutants already present in the innoculum (Farci *et al.,* 1996). The rate of sequence variation in the HVR1 region during chronic HCV infection is lower in the absence of a humoral immune response, as demonstrated for patients with hypogammaglobulinemia (Booth *et al.,* 1998). Furthermore, increased viral diversity in HVR1 is associated with a lack of control of HCV infection (Farci *et al.,* 2000).

D. Human Immunodeficiency Virus

Human immunodeficiency virus (HIV) escape from humoral immunity has recently been reviewed (Parren *et al.,* 1999), and the emergence of neutralization

escape mutants during the course of HIV-1 and simian immunodeficiency virus (SIV) infections has been documented (Albert *et al.,* 1990; Tremblay and Wainberg, 1990; Arendrup *et al.,* 1992; Burns *et al.,* 1993; Moog *et al.,* 1997; Bradney *et al.,* 1999). However, autologous nAb responses against primary isolates during natural HIV-1 infection are usually weak. Passive immunization with HIV Ig and several broadly neutralizing monoclonal antibodies in the experimental chimeric HIV-1/SIV infection of macaques provided protection (Mascola *et al.,* 1999, 2000; Shibata *et al.,* 1999; Baba *et al.,* 2000), indicating that under certain conditions, HIV nAbs are able to provide immunological selection pressure (Poignard *et al.,* 1999).

E. Lymphocytic Choriomeningitis Virus

While a strong $CD8^+$ cytotoxic T cell response is responsible for acute viral clearance of LCMV (Kagi *et al.,* 1994), indirect evidence from B-cell-deficient mice as well as passive immunoprophylaxis studies suggested an important role for nAbs for long-term viral control (Thomsen *et al.,* 1996; Baldridge *et al.,* 1997; Planz *et al.,* 1997). LCMV infection in CTL-deficient mice provided an opportunity to demonstrate direct clearance by nAbs (Ciurea *et al.,* 2000). However, viral control by the humoral response was only transient. The re-emergent virus variants were shown to be neutralization-escape mutants. They displayed one to three single amino acid substitutions within three regions of the envelope glycoprotein-1. One of the amino acid changes had previously been shown to occur following immune pressure by a neutralizing monoclonal GP1-specific antibody (Seiler *et al.,* 1999). All of the substitutions altered neutralization by polyclonal LCMV-immune sera and selected monoclonal antibodies (Ciurea *et al.,* 2000). Interestingly, no new nAb responses against the emerging virus mutants were elicited. This was shown to be the consequence of a time-dependent induction of $CD4^+$ T cell unresponsiveness and therefore lack of T help during high viremia (Ciurea *et al.,* 2001). Interestingly, while the original wild-type strain induced a response against itself but not against emerging variants, the mutant viruses were able to induce nAbs that inhibited both wild-type and escape variants (Ciurea *et al.,* 2000, 2001). Therefore, nAb responses against subsequently emerging LCMV variants seem to recapitulate their evolution in a new sort of co-evolutionarily directed "archetypical" connectivity. This phenomenon is somewhat different from that shown during influenza virus infections in the context of pre-existing immune memory called *original antigenic sin* (Francis *et al.,* 1953; Fazekas de St. Groth and Webster, 1966a, b). In this case, after infection with an escape HA1* mutant (following antigenic drift), influenza HA1-immune individuals will generate higher hemagglutination-inhibiting antibody titers against the original HA1 virus than against the drifted HA1* variant.

X. Immunological Memory

Host and virus represent two sides of an evolutionary equilibrium. Cytopathic agents usually kill immunologically low and late responders, whereas high and early responders tend to survive. Generally, the presence of neither memory B nor T cells will improve these conditions unless antibodies are preexisting or T cells are activated, neither of which are efficiently generated by standard vaccination strategies (reviewed in Ahmed, 1992; Swain and Bradley, 1992; Gray, 1993; Zinkernagel *et al.,* 1996; Ahmed and Gray, 1996; Zinkernagel, 2000a). If an unprimed host survives a first infection, that host will not need immunological memory to survive the second infection. Of course, if the host dies due to the primary infection, immunological memory is, obsolete. Immunological memory, therefore, must confer some evolutionary advantage for the species. This advantage may stem from the fact that maternal immunological memory provides protection for immuno-incompetent newborn vertebrates (Zinkernagel *et al.,* 1996; Zinkernagel, 2000a). Because, MHC-restriction in T cell recognition requires MHC polymorphism, immuno-incompetence of the newborn is necessary to prevent potential host-vs.-graft and graft-vs.-host reactions. In addition to immunodeficiency of the offspring, lack of reactivity is ensured by immunosuppression of the mother and absence of MHC-Ag expression at the maternal–fetal interface (Booy *et al.,* 1992; Sarvas *et al.,* 1992; WHO Study Group, 1995; Brent, 1997; Siegrist *et al.,* 1998). Consequently, protection of the offspring during this critical period is mediated by maternal memory through passive transfer of soluble antibodies. An impressive example of successful transfer of maternal memory is illustrated by the fact that calves are born completely without serum Igs and must take up colostral maternal Igs within the first 24 hours after birth (reviewed by Brambell, 1970). If this does not occur, calves will remain without protective antibody and usually die of various infections during the next few weeks. Maintenance of maternal antibody memory is required to provide the offspring with protection against infectious agents because a complete primary antibody repertoire sufficient to cover all relevant infectious agents cannot be generated during the 270 days of a human pregnancy or the 20 days of a mouse pregnancy. Therefore, B and T cell memory is needed in order to accumulate immunological experience before pregnancy. In addition, hormones may well help to maintain protective antibody levels in the mother by increasing plasma cell survival. This might explain the 5 times higher incidence of auto-antibody-mediated autoimmune disease in females. Therefore, while protection of the newborn is the main and key evolutionary basis for immunological memory there are additional qualities (including herd immunity and individual fitness) that could contribute to a co-evolutionary balanced phenotype.

A. What Kind of Immunological Memory Is Biologically Relevant?

Immune responses and protection against cytopathic virus infections are key to species survival. Without exception, protection against these types of agents is mediated by protective antibodies. Noncytopathic viruses are usually transmitted before or at the time of birth when offspring are immuno-incompetent and this does not have any apparent disadvantage for survival of the species. Examples include LCMV (Rowe, 1954; Hotchin, 1962; Ciurea *et al.,* 1999), mammary tumor virus (MMTV) (Acha-Orbea and Palmer, 1991), and leukemia viruses in mice and hepatitis B virus in humans (Michalak *et al.,* 1994; Rehermann *et al.,* 1996). Although the presence of high titers of nAbs may reduce or prevent transmission of infection from mother to offspring, overall protective immunity is not really necessary for survival of the species. Therefore, taking these considerations into account, it becomes evident that in order to understand protective immunological memory, cytopathic viruses should be studied because it is only here that memory responses are relevant.

B. What Maintains Antibody Memory?

As discussed previously, protection against evolutionarily important lytic virus infections, including those frequently contracted in childhood, is largely mediated by nAbs, although it is well recognized that memory antibody titers tend to decrease over time. Therefore, mechanisms have evolved to help sustain antibody levels. Maternal antibodies have been shown to participate in the deposition of immune complexes on follicular dendritic cells of the offspring which can help maintain antibody memory (Nossal *et al.,* 1965; Tew *et al.,* 1990; Bachmann *et al.,* 1994a). Alternatively, periodical re-infection from either external sources (poliovirus, herpesvirus, influenza, parainfluenza, and many intestinal viruses) or internal foci of persisting infectious agents (HBV, HIV, and the various herpesviruses) provides natural boosters of immunity.

Some viruses do not persist in the host as intact virus particles but in a crippled form (Billeter *et al.,* 1994). For example, measles virus persists not only in subacute sclerosing panencephalitis (SSPE) patients but also apparently in most (if not all) infected hosts (Katayama *et al.,* 1995). This explains why in the classical epidemiological studies on the Faroes or Pacific Islands protective memory was maintained for more than 60 years in previously infected survivors but not in those born on the island after the last epidemic (Mims, 1987).

Two studies have provided evidence for the presence of long-lived bone marrow plasma cells following immunization with ovalbumin or infection with LCMV (Manz *et al.,* 1998; Slifka and Ahmed, 1998). In contrast, extensive analysis of B cell memory and long-term antibody titers post VSV and LCMV infection

indicated that, although memory B cells may be long-lived, antibody secreting plasma cells are short-lived (Ochsenbein *et al.,* 2000b). This study revealed that, in order to maintain long-term antibody titers, continuous antigen-driven and T-cell-dependent differentiation of B cells to plasma cells must occur. How can the differing results of these studies be explained? While the first two studies (Slifka and Ahmed, 1998) analyzed B cell memory of binding antibodies using ELISA assays, Ochsenbein *et al.* studied protective neutralizing antibody responses against replicating and nonreplicating antigens in addition to binding antibodies. As outlined above, protection against a primary infection and/or against re-infection with acute cytopathic viruses largely depends on nAbs (Christian *et al.,* 1996; Bachmann and Zinkernagel, 1997; Bachmann *et al.,* 1997b). Neutralizing antibodies also influence infection with the noncytopathic LCMV (Baldridge and Buchmeier, 1992; Thomsen *et al.,* 1996; Planz *et al.,* 1997) but are irrelevant for ovalbumin. In contrast, the role of non-neutralizing antibodies in host protection is negligible in both LCMV and VSV infections. The discrepancies between these studies may reflect differences in avidities of antibodies which are probably lower for ELISA and higher for neutralization (Bachmann *et al.,* 1997b) and/or perhaps more important differences in the numbers of antigenic sites assessed (one for neutralization and many for ELISA).

After antigen encounter, specific B lymphocytes undergo clonal expansion. This expanded pool of antigen-specific B cells produces a faster and stronger response upon antigen re-encounter. In a recent study, Maruyama *et al.* (2000) generated mice with an inducible genetic switch in the BCR to generate memory B cells in the complete absence of specific antigen. They clearly demonstrated that memory B cells can persist over a long period of time in the absence of any antigenic stimulation. However, as outlined above, from an evolutionary point of view, memory B cells are not sufficient for protective memory. In addition, they cannot differentiate into antibody-secreting plasma cells early enough following re-infection with a cytopathic virus to mount protective antibody titers. Thus, to maintain long-term protective antibody titers, long-lived and antigen-independent memory B cells have to be "reminded" by antigen to differentiate to short-lived antibody-secreting plasma cells (discussed in Ochsenbein *et al.,* 2000b).

C. A Comparison with T Cell Memory

While T cell memory is not the focus of this review, it is interesting to note some important differences with B cell memory. Cytotoxic T cells have the key function of controlling noncytopathic viruses during acute infection. Although protective during this period, they may also be harmful to the host because they can cause immunopathological destruction of infected cells and therefore must be controlled (reviewed in Zinkernagel *et al.,* 1996). For example, a lethal

graft-vs.-host-like immunopathology can be induced by injection of a high dose of viral peptide into a primed mouse that has a high precursor frequency of virus-specific CTLs. The immunopathology probably occurs as a result of peptide loading of many host cells, including cells of the immune system which are then killed by primed CTLs (Oehen *et al.,* 1991; Aichele *et al.,* 1997). Therefore, while memory nAb responses are required to protect against cytopathic infections, CTLs protect against noncytopathic acute infections, and increased precursor frequencies may actually be harmful.

In contrast to maternal antibodies, CTL responses are not transmitted to offspring because transplantation antigen differences between mother and offspring can potentially cause graft-vs.-host reactions. In addition, the specificity of maternal T cells will not recognize the paternal MHC–peptide configuration of the offspring and would therefore be useless. Therefore, primed CTLs may function primarily to prevent virus from spreading again within the same host, so as to limit or prevent immunopathological disease (Kundig *et al.,* 1996; Bachmann *et al.,* 1997c). An example illustrating this point is the spectrum of virus–host relationships found after HBV or HIV infections in humans. If virus is controlled down to low levels, then chronic disease either does not develop or develops only very late. However, if the virus is not controlled, a severe autoaggressive disease (aggressive form of HBV-hepatitis) may develop. A similar balance exists in lepra or tuberculosis infections. In all these examples low-level infection maintained protective immunity (reviewed in Bianchi, 1981; Mondelli and Eddleston, 1984; Chisari and Ferrari, 1995). Mackaness introduced the term *infectious immunity*, or infection immunity, to describe this important co-evolutionary equilibrium (Mackaness, 1964, 1969).

We conclude that although immunological memory has been considered to be an indispensable component of an individual's immune system, this may be more idea than fact. Protective immunological memory most likely reflects a low-level response driven and maintained by persisting or re-encountered antigen. An antigen-driven memory response would protect the host against both direct damage by infections and indirect (immunopathologically mediated) damage mediated by infectious agent-specific T cells and antibodies. Most importantly, from an evolutionary point of view, antibody memory protects offspring during the physiological phase of immunodeficiency.

XI. Neutralizing Antibodies and Vaccine Strategies

When we consider successful vaccines and compare them with those infectious diseases where efficient protective vaccines are lacking (reviewed in Nossal, 1998), it is striking to note that all successful vaccines induce high levels of nAbs that are both necessary and sufficient to protect the host from disease. Successful vaccination against infectious diseases such as tuberculosis, leprosy,

or HIV would require induction of additional long-lasting T cell responses to control infection. Although long-lasting nAb responses can be efficiently generated by classical vaccination strategies we are not yet able to generate vaccines that mimic infections that persist at very low levels, providing the necessary long-term T cell immunity.

A. Successful Vaccines

Generation of neutralizing antibodies is central to successful vaccine development. Poliovirus vaccines serve as a good example (Nathanson and Martin, 1979; Sabin, 1985). Neutralizing antibodies in the form of mucosal IgA, or serum IgG, can prevent or reduce infection at a very early stage. Both the Sabin and the inactivated Salk vaccine induce long-lasting IgG responses in serum that prevent circulating virus from reaching the central nervous system. Since none of the successful vaccines is able to completely prevent re-infection, the generally accepted mode of action is to greatly reduce systemic spread of the infectious agent through induction of nAbs.

B. Ineffective Vaccines

Analysis of successful vaccine strategies may perhaps reveal why certain vaccines, including mumps (Arya, 1994; Strohle *et al.,* 1997) and measles (Ofosu-Amaah, 1983; Garenne *et al.,* 1991; Malfait *et al.,* 1994; Wild, 1999; Bennett *et al.,* 1999), both of which are paramyxoviruses, are less effective and therefore subject to criticism by opponents of vaccination. Mumps vaccines cannot always prevent re-infection of the salivary gland (Germann *et al.,* 1996; Strohle *et al.,* 1997); however, severe forms of disease such as orchitis and encephalitis do not occur in vaccinated children. This finding may indicate that, following systemic vaccination with a low-dose vaccine, local mucosal immunity may not be sufficiently high enough to protect against re-infection. However, similar to the Sabin vaccine, even though a local breakthrough can occur in the mucosa, systemic disease does not develop because virus is trapped by neutralizing IgG in the blood.

Similar considerations may apply to measles vaccines, although the consequences of re-infection are more severe. Once again, systemic measles immunization may not provide sufficient titers of local antibodies to prevent infection of throat and tonsils. In addition, measles virus has the capacity to cause immunosuppression by interfering with APC and B cell function (reviewed in McChesney and Oldstone, 1989. Interestingly, this may also apply to the attenuated vaccine strains. Increased incidences of insufficient vaccine protection, together with the possibility that maternal antibodies may interfere with efficient vaccination during early childhood (Sabin *et al.,* 1983; Englund *et al.,* 1998; Siegrist *et al.,* 1998), are of great concern, particularly for third-world countries (Gwatkin, 2000). In an attempt to enhance measles vaccine efficacy, the innoculum dose was increased

by a factor of 10 to 100, but this led to late disease development in children and therefore has been stopped (Gwatkin, 2000). This example shows that even attenuated viruses, when given in high doses, can cause disease comparable to low-dose infections with wild-type viruses. The details of these complications with measles are still unknown and require further evaluation. Nevertheless, this example demonstrates that attenuation of a virus is not going to yield optimal vaccines in all instances. Wild-type measles is also interesting because it may persist in the host for a very long time, possibly until death (Katayama *et al.*, 1995). Whether this also applies to vaccine strains is not yet known. However, it is important to determine where measles virus persists and for how long. In addition, whether such persisting virus is necessary and sufficient to maintain long-term protection and whether vaccine strains can also cause SSPE must be investigated.

C. Attenuated Vaccines that Provide Insufficient Protection or Cause Disease

Whether or not attenuated viruses are always good vaccine candidates is debatable. This question has recently been discussed using naturally occurring nef-deficient HIV in humans and attenuated SIV strains in monkeys as examples (Ruprecht, 1999). Although both attenuated strains did not initially cause immunodeficiency, eventually both humans and monkeys developed disease and some of them died.

Similarly to HIV infection, successful long-term protective vaccines against tuberculosis or leprosy are still not available (Bloom and Ahmed, 1998; Bloom and McKinney, 1999). In contrast to wild-type tuberculosis, bacillus Calmette-Guerin (BCG) apparently does not persist in humans for more than a few years and this correlates with the time of protection against tuberculosis in young children. This suggests that persistent infection is required for maintenance of an activated T cell response which limits granuloma size in order to avoid immunopathology yet sustains long-lasting immune responses (Bloom and McKinney, 1999).

In summary, attenuated vaccines strains cannot yet imitate that optimal mixture of "infection immunity" as defined by Mackaness (Mackaness, 1962, 1969). Similarly, when CTL responses are necessary to control infection, such as for HIV, HCV, and herpesviruses, attenuated viruses have not been successful. At this time, DNA vaccination may be the most promising candidate to provide a low-dose persistent antigen source that guarantees long-term activation of both T and B cells.

D. Linear Epitopes for Induction of Neutralizing Antibodies

A discussion of vaccine strategies should also include the old hope of mimicking neutralizing epitopes using linear peptides that would provide a safe, nonreplicating vaccine that induces long-lasting titers of nAbs. Theoretically, such a

strategy should be successful and has been shown to be effective in rare examples such as with foot-and-mouth disease virus (Bittle *et al.,* 1982). In addition, use of linear peptides of the V3 loop of HIV has provided limited success *in vitro* but has not yet yielded success *in vivo* (Conley *et al.,* 1994). In general, it is very difficult to imitate the serotype-specific three-dimensional structure of neutralizing epitopes. This is particularly true for high avidity-neutralizing antibody/antigen interactions. The reasons probably rest in the simple fact that neutralizing epitopes are, in general, not linear but are composed of two or more loops of protein chains.

XII. Conclusions

Studies of neutralizing antibodies against viruses, bacteria, and toxins have largely been neglected in the past 30 years. However, numerous studies have focused on induction of binding antibodies against soluble protein antigens such as ovalbumin or against haptens. Although these classical studies have provided some insights into B cell and antibody function, re-evaluation of the conclusions reached is timely and necessary. One major reason for neglecting to study antiviral antibody responses has been perhaps that T-cell-mediated immunity occupied much of the interest of immunologists. Recently, many new tools have been developed to allow analysis of B cell and neutralizing antibody responses at a level that was not imaginable 30 years ago. Antibodies can now be sequenced rapidly, transgenic and gene-knockout mice permit the evaluation of precise immunological functions, and advances in molecular virology provide many new and fascinating research perspectives.

Neutralizing antibodies are evolutionarily important effectors of immunity against viruses. Their evaluation has revealed a number of basic insights into specificity, rules of reactivity (tolerance), and memory: (1) Specificity of neutralizing antibodies is defined by their capacity to distinguish between virus serotypes; (2) B cell reactivity is determined by antigen structure, concentration, and time of availability in secondary lymphoid organs; and (3) B cell memory is provided by elevated protective antibody titers in serum that are depending on antigen stimulation. These perhaps slightly overstated rules are simple, correlate with *in vivo* evidence as well as clinical observations, and appear to largely demystify many speculations about antibodies and B cell physiology. The implications for vaccines are obvious; therefore, careful and critical assessment of the reviewed experimental evidence and concepts will be of great importance.

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