

Structural and functional studies on a unique linear neutralizing antigenic site (G5) of the rabies virus glycoprotein

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The core of a unique linear neutralization epitope (G5) on the glycoprotein of rabies virus, recognized by a virus-neutralizing mouse monoclonal antibody (MAb 6-15C4), was determined by Pepscan analysis. The G5 epitope was defined as an octapeptide (LHDFRSDE). The contribution of the individual amino acids of the G5 epitope to the binding of MAb 6-15C4 was analysed with a set of synthetic peptides in which the individual amino acids had been replaced in turn by each of the other 19 naturally occurring amino acids. Five amino acids of the octapeptide proved to be essential for the binding of MAb 6-15C4. The conservation of the G5

epitope within the glycoprotein of the different rabies virus strains sequenced to date proved to be absolute at the amino acid level. Studies concerning the immunodominance of the G5 epitope were carried out by determining the presence of G5 epitope-specific serum antibodies in vaccinated humans and mice, and by determining the frequency of G5 epitope-specific B lymphocytes in the blood of vaccinated humans. These studies indicated that antibodies to the G5 epitope constitute a minor population of the rabies virus-specific serum antibodies induced by rabies vaccination.

Introduction

Protective immunity against rabies is mediated by several host-effector mechanisms of which virus-neutralizing (VN) antibodies are a major constituent (Sikes *et al.*, 1971; Turner, 1985; Dietzschold *et al.*, 1989). The presence of VN antibody may give complete protection against rabies virus infection, as shown by transfer of VN antibodies before exposure (Dietzschold *et al.*, 1990a). Therefore, the induction of VN antibodies is one of the primary goals in the generation of protective immunity against rabies by vaccination. VN antibodies are solely directed against the glycoprotein of the virus (Sikes *et al.*, 1971; Wiktor *et al.*, 1984). At least six epitopes on the glycoproteins of different rabies virus strains, responsible for the induction of VN antibodies, have been identified using VN mouse monoclonal antibodies (MAbs). With one exception these antigenic determinants are not recognized by their corresponding MAbs when the glycoprotein is denatured (Bunschoten *et al.*, 1989; Dietzschold *et al.*, 1990b). This suggests that the natural conformation of the protein is required for their recognition. Recently we have described a unique epitope (G5) on the glycoprotein of rabies virus which is recognized by MAb 6-15C4 (Bunschoten *et al.*, 1989).

This epitope was not dependent on the native conformation of the glycoprotein indicating that the G5 epitope is linear (Dietzschold *et al.*, 1990b). The antigenic site to which the G5 epitope maps has been tentatively identified by sequence analysis of the glycoprotein of escape mutants, resistant to neutralization by MAb 6-15C4. One amino acid substitution in the G5 epitope (arginine to histidine at position 264) correlated with lack of neutralization (Dietzschold *et al.*, 1990b). A 21 amino acid synthetic peptide (G5-21), spanning the G5 epitope recognized by MAb 6-15C4 induced VN antibodies *in vivo* (Dietzschold *et al.*, 1990b). When used in tandem with a peptide representing a dominant T cell epitope on the nucleoprotein of rabies virus, a significant protective immune response could be induced in C₃H mice against a lethal rabies virus challenge. From these data it was speculated that a completely synthetic vaccine against rabies could be produced (Dietzschold *et al.*, 1990b).

So far the core of the G5 epitope and the degree of conservation of this apparently important antigenic determinant on the rabies virus glycoprotein have not been identified. Our interest in the structure of this site is not based solely on its potential as a linear epitope that may provide a basis for a synthetic vaccine, but also on

the possibility of identifying a structural homology with a recently generated human MAb, MAb 383, which recognizes the paratope of MAb 6-15C4 (van der Heijden *et al.*, 1991). The Epstein-Barr virus (EBV)-transformed CD5⁺ B cell (EBV 383) producing MAb 383 was derived from a rabies virus-vaccinated individual and we reasoned that it had arisen in the course of an idiotype-driven immune response (van der Heijden *et al.*, 1990, 1991), a mechanism originally proposed by N. Jerne in his immune network theory (Jerne, 1974). Our speculation was based in part on the observation that the recognition of the 6-15C4 idiotype by its anti-idiotype MAb 383 was inhibited by rabies virus and by the G5-21 peptide (van der Heijden *et al.*, 1991).

Here we describe the identification of the core of the G5 epitope by Pepscan analysis, the contribution of individual amino acids to the binding of MAb 6-15C4, the conservation of the epitope among different rabies virus strains and the degree of immunodominance of the epitope in the specific antibody response following rabies vaccination.

Methods

Rabies VN MAb (MAb 6-15C4). The generation and characterization of MAb 6-15C4 has been previously described (Bunschoten *et al.*, 1989). It was generated by fusion of P3-X63-Ag8.653 cells with spleen cells from BALB/c mice that had been immunized with rabies virus (Pitman-Moore strain) antigen. MAb 6-15C4 was purified from tissue culture supernatant by Protein A-Sepharose affinity chromatography (Pharmacia), and stored at a concentration of 4 mg/ml at -20 °C. Isotype determination (IgG2b, κ), VN activity and the identification of a linear epitope (G5) on the rabies virus glycoprotein recognized by MAb 6-15C4 have been described elsewhere (Bunschoten *et al.*, 1989; Dietzschold *et al.*, 1990b).

Mouse MAbs directed against other antigenic sites of the glycoprotein of rabies virus (Wiktor & Koprowski, 1978; Flamand *et al.*, 1980; Lafon *et al.*, 1983, 1984) were kindly provided as concentrated culture supernatant by Dr B. Dietzschold.

Pepscan and amino acid replacement studies. Peptides were synthesized on polyethylene rods and tested for binding with MAb 6-15C4 in an ELISA according to established procedures (Geysen *et al.*, 1984, 1985). A set of overlapping peptides of between three and 15 residues with sequences corresponding to the rabies virus glycoprotein region between amino acids 254 and 275, and a set of octapeptides, LHDFRSDE, in which each amino acid was substituted by the other 19 naturally occurring amino acids, were synthesized.

Rabies virus glycoprotein sequences. The nucleotide sequences of the genes encoding the glycoproteins from different rabies virus strains have been previously described (Anilionis *et al.*, 1981; Tordo *et al.*, 1986; Morimoto *et al.*, 1989; Conzelman *et al.*, 1990; Benmansour *et al.*, 1992). Sequence files were obtained from the EMBL/GenBank databank. Primary amino acid sequences were deduced and alignments were carried out using the DNA star program (DNASTAR Inc.).

Pre- and post-vaccination sera. Serum samples were collected from human individuals before and at different times after the start of a series of three vaccinations with the dog kidney cell vaccine (DKCV)

against rabies produced by the National Institute of Public Health and Environmental Protection. This vaccine is based on the Pitman-Moore strain of rabies virus (van Wezel *et al.*, 1978). Serum samples were also collected from 30 outbred NIH mice, vaccinated with the same vaccine according to the same schedule. All serum samples were tested for the presence of rabies VN antibodies in the rapid focus-forming immunofluorescence test as described by Wiktor & Koprowski (1978).

Inhibition ELISA. Serum antibodies present in polyclonal anti-rabies virus antibody preparations that interfered with the binding of MAb 6-15C4 to its corresponding G5 epitope were detected with an inhibition ELISA essentially as previously described for another virus system (Groen *et al.*, 1992). Briefly, ELISA plates (Costar) were coated with 6 μ g/ml inactivated rabies virus (Pitman-Moore strain) antigen (van Wezel *et al.*, 1978). The plates were incubated with 10-fold dilutions of the sera to be tested. After incubation for 1 h at 37 °C half of the serum was replaced by an equal volume of Protein A-Sepharose-purified MAb 6-15C4 at the optimal dilution and incubated for 1 h at 37 °C. A horseradish peroxidase (HRP)-conjugated rabbit anti-mouse immunoglobulin (Dakopatts) was used to detect MAb 6-15C4.

For the inhibition with the panel of rabies virus-specific mouse MAbs, biotin-labelled MAb 6-15C4 was used in combination with streptavidin-HRP. Plates were developed as previously described (Groen *et al.*, 1992). Percentage inhibition was defined as the percentage inhibition of the maximal reactivity obtained without inhibiting antibody.

Peptide ELISA. Peptide G5-21 was synthesized on *p*-alkoxybenzyl alcohol resin, purified and characterized as described earlier (Bunschoten *et al.*, 1989). Microtitre plates were coated with 0.5 μ g peptide/well in PBS (pH 7.2) for 18 h at room temperature. After adding a 200-fold dilution of human serum, plates were incubated for 1 h at 37 °C and binding of human Ig was detected with HRP-conjugated rabbit anti-human Ig (Dakopatts). Mouse serum antibodies directed against the G5-21 peptide were determined with serum samples diluted 100-fold in the same assay, using a HRP-conjugated swine anti-mouse Ig preparation (Dakopatts). The results of each serum were expressed as the difference between the A_{450} obtained with pre- and post-vaccination serum ($\Delta G5$). The same sera were always tested in parallel in a similar assay, using an irrelevant 21 amino acid peptide coat (Δip).

ELISA spot test. To detect human B cells producing rabies virus-specific immunoglobulins or anti-G5 immunoglobulins *in vitro*, we used an ELISA spot test essentially as described previously (UytdeHaag *et al.*, 1985). Briefly, peripheral blood mononuclear cells (PBMCs) isolated from rabies-vaccinated human individuals were stimulated *in vitro* with 5 μ g/ml rabies virus antigen and cultured for 3 days. The cells were washed and titrated on microtitre plates coated with 6 μ g/ml rabies virus antigen or 5 μ g/ml peptide G5-21. After incubation at 37 °C overnight, plates were washed and incubated with alkaline phosphate-conjugated goat anti-human immunoglobulin (Tago) and subsequently developed as described earlier (UytdeHaag *et al.*, 1985). Blue coloured spots were visible after overnight incubation at 4 °C. The MAb 6-15C4-producing hybridoma was used in both spot tests as a positive control.

Results

Pepscan with overlapping peptides of variable length

To define the core of the G5 epitope, the reactivity of MAb 6-15C4 with overlapping peptides of the G5 epitope (amino acids 254 to 275), varying in length from three to 15 amino acids, was determined by indirect

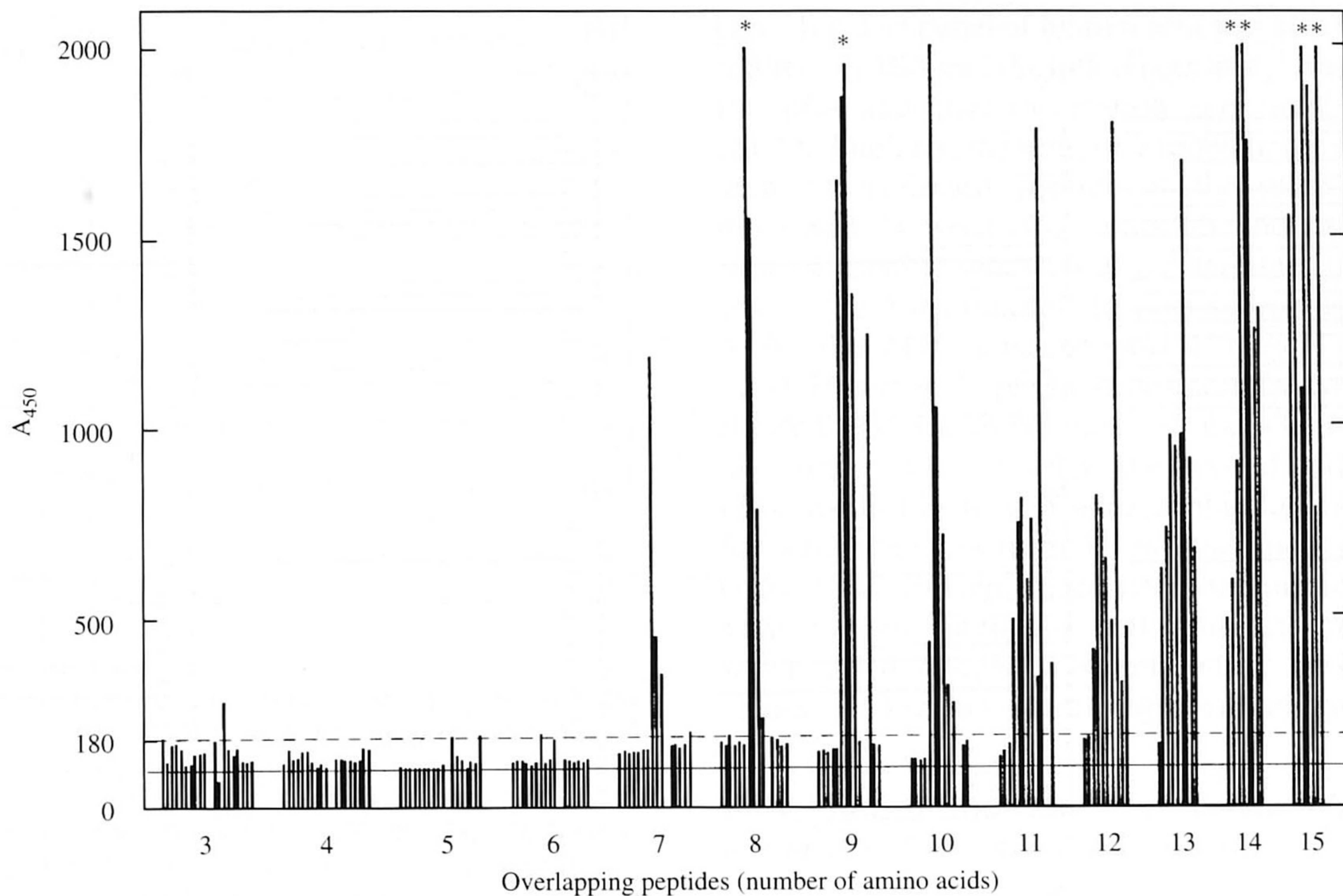


Fig. 1. Pepscan with overlapping peptides of different lengths and MAb 6-15C4 (100-fold dilution). The amino acid sequence of the G5-21 peptide (P_{254} DQLVNLHDFRSDEIEHLVVE E_{275}) was derived from the nucleotide sequence of a rabies virus glycoprotein (Dietzschold *et al.*, 1990*b*). Overlapping peptides were between three and 15 amino acids in length, starting at P_{254} and were moved, one amino acid in turn, towards the E_{275} position (e.g. three amino acid peptides PDQ, DQL, QLV, LVN, etc.). Binding reactivity of each peptide is indicated as a vertical bar proportional to the measured A_{450} . The asterisks (*) indicate $A_{450} > 2000$.

ELISA. As shown in Fig. 1, the heptapeptide HDFRSDE was the smallest peptide binding to MAb 6-15C4. A higher reactivity was obtained with the octapeptide, LHDFRSDE. Reactivities with the subsequent octapeptides, HDFRSDEI, DFRSDEIE, etc., were lower or absent. Reactivities with longer overlapping peptides containing the LHDFRSDE sequence were not significantly higher and the absence of this complete sequence in larger peptides substantially reduced or abolished the reactivity of MAb 6-15C4. The octapeptide LHDFRSDE (G5-8) was therefore defined as the core of the G5 epitope.

Irreplaceable amino acids in the G5 epitope

Amino acid replacement studies were performed to define the amino acid residues in the G5-8 epitope (LHDFRSDE) essential for the binding to MAb 6-15C4. Each amino acid of the original octapeptide was substituted in turn by all 19 naturally occurring amino acids. If replacement by most of the 19 naturally occurring amino acids resulted in a significant or complete loss of MAb 6-15C4 binding, we concluded that the original amino acid was essential for binding of MAb 6-15C4. The reactivities of these peptides with MAb 6-15C4, demonstrated by indirect ELISA, are

shown in Fig. 2. Five out of eight amino acids at different positions in the peptide were important for binding MAb 6-15C4: H_2 , D_3 , F_4 , R_5 and E_8 . Amino acid R_5 is probably the most essential, since it could not be substituted by any other amino acid without completely abolishing reactivity with MAb 6-15C4, even when 10-fold higher MAb concentrations were used (not shown). Two amino acids that were replaceable (L_1 and D_7) could be substituted by other amino acids that improved MAb 6-15C4 binding as compared to the original residues: substitution of L_1 by amino acids P, Q or V and of D_7 by amino acids A or G caused a significant increase of MAb 6-15C4 reactivity. Since A and G are the smallest amino acids, it may be postulated that the D_7 position probably has a 'spacer' function. Substitution of amino acid L_1 by amino acids with a negative charge (D and E) resulted in a substantial loss of antibody binding. Similarly, substitution of amino acid S_6 by amino acids with a negative charge or by hydrophobic amino acids caused loss of antibody binding.

Conservation of the G5 epitope in rabies virus strains

The published nucleotide sequences encoding the G5 epitope (nucleotides 852 to 875) of different rabies virus strains were compared (Fig. 3). The sequences of the

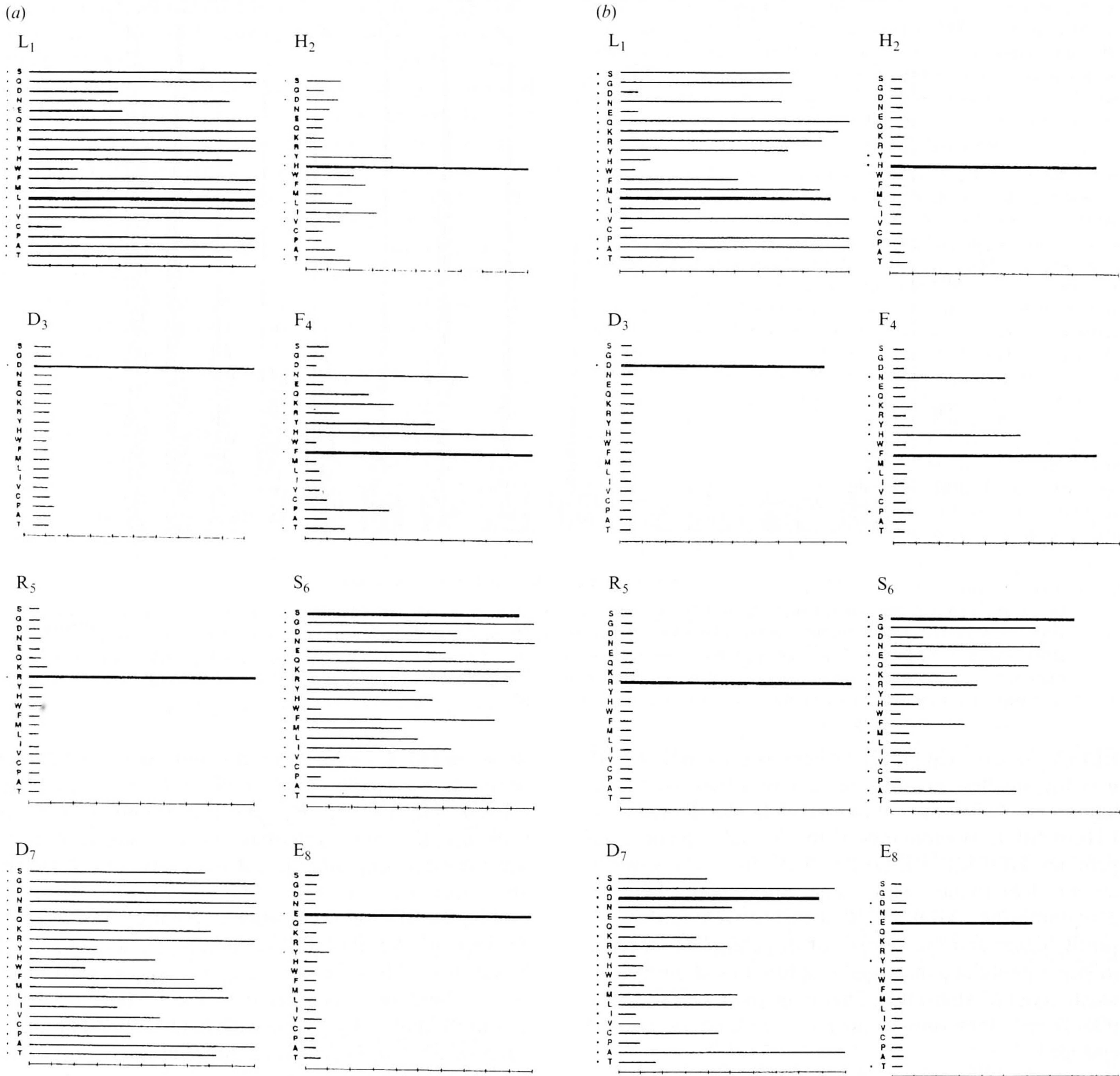


Fig. 2. (a) Pepsan with replacement peptides based on the G5-8 octapeptide amino acid sequence (LHDFRSDE) and MAb 6-15C4 (1000-fold dilution). Binding reactivity of each peptide is indicated as a horizontal bar proportional to the measured A_{450} . Amino acid replacements are indicated on the y-axis. Every group of 20 lines corresponds to the complete replacement set for each amino acid position in the G5-8 octapeptide. Within each group of 20 horizontal bars, the substitution of the original amino acid is in the order SGDNEQKRYHWFMLIVCPAT, according to the arrangement described by Doolittle (1985). (b) Same as (a), differing only in dilution of MAb 6-15C4 ($1:10^4$).

ERA, CVS and SAD strains were identical in this region, whereas in the corresponding sequences of the other five strains tested, one, two or three nucleotide differences were observed. None of these substitutions resulted in differences at the protein level in the G5 epitopes of the respective rabies virus strains. This shows that the

epitope is highly conserved among the rabies virus strains studied, which is consistent with the observation that in an indirect ELISA or immunofluorescence assay, MAb 6-15C4 reacts with all rabies virus strains tested including the ERA, CVS, Flury HEP and Pasteur (Pitman-Moore) strains (not shown).

Rabies virus strain	Nucleotide position (852–875)							
	CTG	CAC	GAC	TTT	CGC	TCA	GAC	GAA
ERA								
CVS	---	---	---	---	---	---	---	---
SAD	---	---	---	---	---	---	---	---
Pasteur	T--	---	---	---	---	---	---	---
Flury HEP	T--	---	---	---	---	---	---	--G
Street (dog)	---	---	---	---	---	--G	--T	--G
Street (human)	---	---	---	---	---	--G	--T	--G
Street (cell-adapted)	---	---	---	---	---	--G	--T	--G
Encoded amino acid	L	H	D	F	R	S	D	E

Fig. 3. Nucleotide sequence comparison of the triplets encoding the octapeptide G5-8 (LHDFRSDE), being the core of the rabies VN G5 epitope. Nucleotide sequences were obtained from the EMBL/GenBank Database and have been described previously (ERA, Anilionis *et al.*, 1981; SAD, Conzelman *et al.*, 1990; Pasteur, Tordo *et al.*, 1986; Flury HEP, Morimoto *et al.*, 1989; CVS and street strains, Benmansour *et al.*, 1992). Triplets encoded for the amino acids given at the bottom of the figure, which were completely identical to the amino acid sequence of the octapeptide G5-8.

G5 epitope-specific antibodies in response to rabies vaccination measured by ELISAs

Serum samples from human individuals, collected before and at different intervals after the start of a series of three vaccinations with the DKCV rabies vaccine, were tested in the inhibition ELISA using rabies virus antigen-coated plates and MAb 6-15C4 as homologous inhibitor. The pre-vaccination sera were all negative in the rabies virus VN assay. In all the post-vaccination sera VN antibodies could be demonstrated (not shown). As shown in Fig. 4(a) the majority of these individuals developed serum antibodies against the G5 epitope, detectable in the inhibition ELISA within 3 weeks after the first vaccination. The percentage inhibition increased at week 6 after the first vaccination and subsequently decreased gradually. MAb 6-15C4 gave a 95% inhibition in this assay when 2.5 µg of Protein A-Sepharose-purified MAb per ml was used (Fig. 4b). Since we had previously shown that MAbs directed to antigenic sites II, III and to unclassified sites may hinder the binding of MAb 6-15C4 (Bunschoten *et al.*, 1989), we tested 21 MAbs of these specificities in the same assay. The results, shown in Fig. 4(b), indicate that these MAbs may indeed cause significant inhibition in this assay up to 75%. Testing of this same panel of MAbs in the indirect ELISA with G5-21-coated plates showed that none of these antibodies recognized the G5 epitope (titres < 100), whereas MAb 6-15C4 reacted strongly (titre > 12500) in this ELISA

(Fig. 4c). The panel of human sera was also tested in this indirect ELISA and the differences in A_{450} values between the pre- and post-vaccination sera were determined ($\Delta G5$). Similarly, the sera were tested in the same ELISA with an irrelevant peptide of the same length and differences between A_{450} values obtained with pre- and post vaccination sera were also determined (Δip). All but one of the Δip values of the post-vaccination sera were < 50. The $\Delta G5$ values of most of these sera were also < 50. However, 13 of the 42 post-vaccination sera tested showed $\Delta G5$ values between 131 and 432. All these sera had been collected after the second vaccination as indicated in Fig. 4(a). The determination of $\Delta G5$ values for serum samples from 30 outbred mice (strain NIH), collected at different times after immunization with the same vaccine, indicated that none of these animals, which all developed VN antibodies, developed $\Delta G5$ values > 50 within 4 weeks after vaccination.

Rabies virus-specific antibody-producing B lymphocytes in PMBCs of vaccinated individuals

The frequency of B cells producing rabies virus-specific antibodies, among the PBMCs of the human donor from whom EBV 383 had been generated, was determined in an ELISA spot test, 11 weeks after he had been re-vaccinated. In this test inactivated rabies virus or the G5-21 peptide were used as antigens. The PBMCs were freshly isolated and used directly in the test or after a 3 day *in vitro* stimulation period with inactivated rabies virus. In unstimulated PBMCs the frequency of rabies virus-specific B cells was approximately 1:10⁴. After *in vitro* stimulation with rabies virus antigen the frequency increased to approximately 1:150. Upon analysis of the same cell suspensions in an ELISA spot test using the G5-21 peptide as a coat, no antibody-producing B cells could be identified.

Discussion

In the present paper, we have defined by Pepscan analysis the octapeptide LHDFRSDE (G5-8) as the G5 epitope on the glycoprotein of rabies virus. By replacing the individual amino acids and determining the binding with its corresponding MAb 6-15C4, the amino acids essential for this binding were determined. Amino acid R₅ was revealed to be the most essential in this respect and may therefore be expected to make the greatest contribution to the binding. Changes in two amino acids (L₁ and D₇) resulted in a higher reactivity of MAb 6-15C4, a finding which may be of interest if this peptide were to be used as part of a synthetic vaccine. The deduced amino acid sequence of the G5 epitope proved to be completely conserved in the glycoproteins of all the

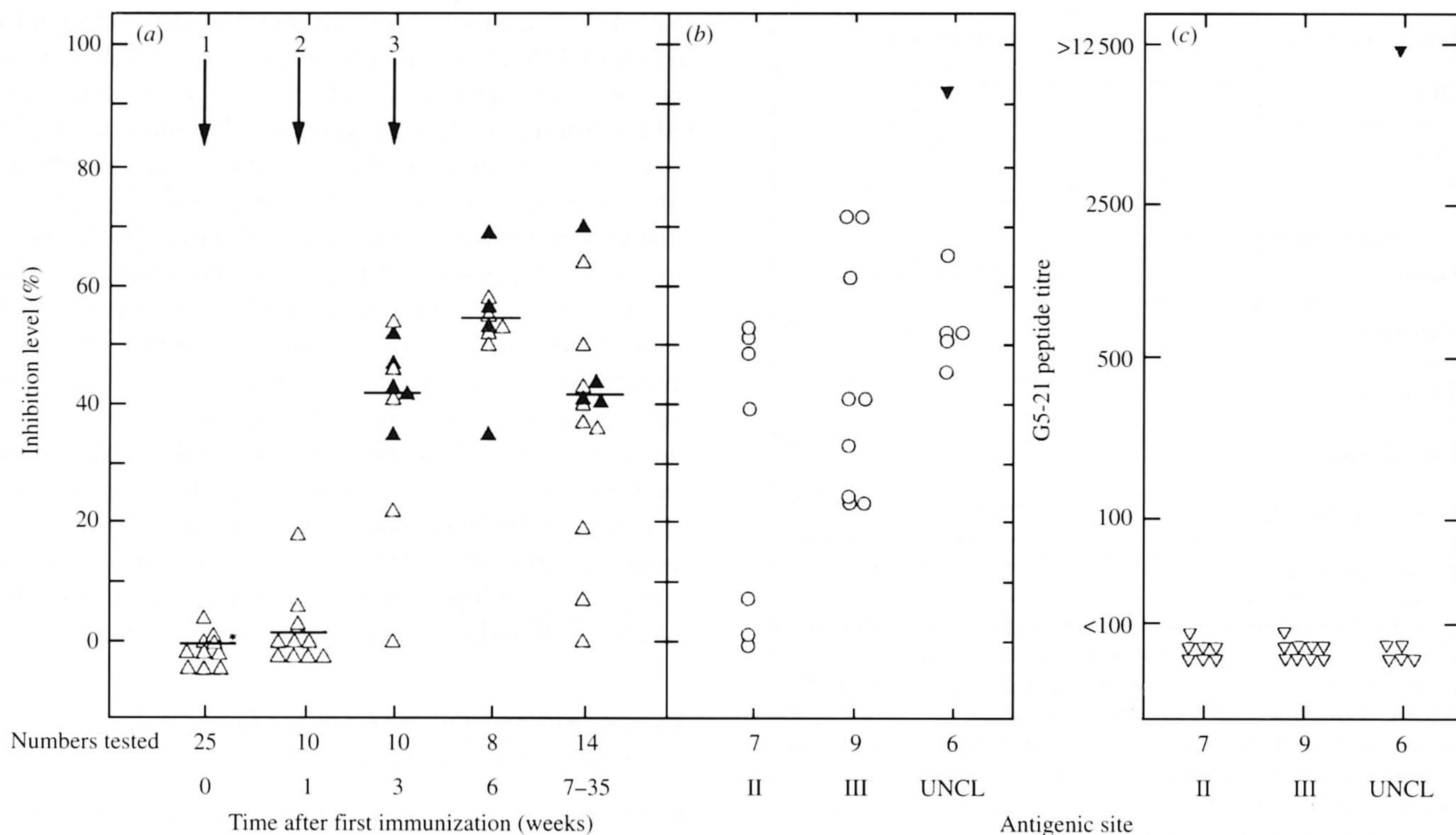


Fig. 4. Binding of the G5 epitope-specific VN MAb 6-15C4 to a rabies virus antigen coat was inhibited and expressed as a percentage after preincubation with (a) 10-fold diluted human anti-rabies virus sera (Δ) (\blacktriangle ; inhibition of binding after preincubation with human anti-rabies virus sera displaying a Δ G5 titre ≥ 132); (b) mouse MAb directed to different antigenic sites of the rabies virus glycoprotein (\circ). (c) Binding of the mouse MAb [introduced in (b)] to a G5-21 peptide coat in an indirect ELISA (∇). Arrows indicate time of immunization. In (b) and (c), MAb 6-15C4 titres are indicated as a filled triangle (\blacktriangledown).

rabies viruses for which the relevant nucleotide sequences have been published. Since these viruses mainly originate from different geographical areas this finding suggests that little immunological pressure is exerted on the G5 B cell epitope by naturally induced VN antibodies directed against this epitope. This is in agreement with the low levels of G5 epitope-specific VN antibodies found in the serum of individuals vaccinated against rabies. The majority of the antibody reactivity detected with the MAb 6-15C4 inhibition ELISA was most likely based on steric hindrance by antibodies directed against other antigenic sites on the glycoprotein of the virus. This was supported by showing that mouse MAbs directed against other sites can cause up to 75% inhibition in this assay, without showing any reactivity with epitope G5 (Fig. 3). Thirteen of the 42 post-vaccination serum samples did exhibit a Δ G5 ranging from 131 to 432, but this is a low number and represents low specific activity. The absence of epitope G5-specific circulating B cells from a revaccinated individual, as demonstrated in the ELISA spot test, confirmed this finding. Also the absence of peptide G5-21-specific antibody responses in mice, exhibiting rabies virus-specific neutralizing serum antibodies, indicates that the antibodies against this epitope are not a major part of the VN antibodies induced by rabies

vaccination. The relative lack of immunogenicity of this epitope may be the reason why it has been so well conserved among different rabies virus strains. The question then arises of why we succeeded in generating a mouse MAb against this epitope in the first place (Bunschoten *et al.*, 1989). However, so far no other groups have generated a MAb against this unique linear site and, despite numerous attempts, we have never succeeded in generating a second mouse MAb of the same specificity (unpublished observation). It may be considered even more surprising that we have been able to generate a human EBV-transformed B cell line producing a human MAb recognizing the idiotope on MAb 6-15C4. Since the binding of MAb 383 to the idiotope of MAb 6-15C4 can be efficiently inhibited by rabies virus antigen and by peptide G5-21 (van der Heijden *et al.*, 1991), B cell clones with these two specificities have probably been expanded in response to rabies vaccination. However, in the donor from whom EBV 383 was generated, no serum antibodies to the G5 epitope (Δ G5 < 50) and no epitope G5-specific B cells could be demonstrated. Therefore, it is possible that, as in other vaccinees, B cells of this specificity have been expanded in this donor only to very low numbers. The identification of an octapeptide (G5-8) as the neutral-

ization epitope in this postulated idiotype-anti-idiotype cascade (van der Heijden *et al.*, 1990, 1991) provides an important tool in determining whether a structural analogue of this G5-8 epitope exists on the anti-idiotypic MAb 383. Analysis of the deduced primary amino acid sequence of the V_H and V_L segments of MAb 383, which we have recently determined (van der Heijden *et al.*, 1990), did not reveal a significant linear sequence similarity to the G5 epitope. Further studies are in progress to identify by three-dimensional computer analysis, a discontinuous idiotope on MAb 383 that mimics the G5 epitope.

It is not clear why the G5 epitope is so poorly immunogenic after vaccination with conventional inactivated whole rabies virus vaccines and probably also after natural infection. The poor immunogenicity is also suggested by the high degree of conservation of this epitope in different rabies virus strains (Fig. 3). However the potential of this epitope as a component of a synthetic rabies vaccine was demonstrated by the induction of VN antibodies and protection of mice after immunization with the G5-21 peptide alone or in combination with a T cell epitope, respectively (Dietzschold *et al.*, 1990b).

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