Volume 292, number 1,2, 148–150 FEBS 10352 November 1991

ADONIS 001457939101074I

Iodoacetamide treatment of bovine leukemia virus glycoprotein gp51 enhances the Western blotting reactivity of anti-peptide antibodies

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Received 12 August 1991; revised version received 17 September 1991

Rabbit polyclonal antibodies were raised against synthetic peptides of the bovine leukemia virus envelope glycoprotein gp51 and tested against the full size protein by the Western blotting technique. We show that acetylation of gp51 by iodoacetamide either maintains or significantly increases the antigen–antibody reaction and conclude therefrom that the reactive potential of an anti-peptide antibody may require acetylation of the sulfhydryl groups of the blotted protein.

Iodoacetamide; Bovine leukemia virus; Western blotting; Anti-peptide; Glycoprotein; Enhancement

1. INTRODUCTION

Synthetic peptides are commonly used to identify and characterize antigenic determinants of protein antigens recognized by the cellular and humoral compartments of the immune response. The strategy was applied here to the external glycoprotein BLV-gp51 [1,2].

Our study involved the assessment of the reactivity of anti-peptide antibodies with the full size protein in serological tests such as ELISA, RIA and WB. We mostly focused attention on the reactivities observed in WB, reasoning that a positive reaction implies that the target amino acid sequence is accessible and that the recognized structure is a sequential epitope. We show here that antigenic determinants of the polypeptide backbone are not necessarily available to the antibody even after WB. The reactivity of some antibody molecules sharply increased after reduction of the protein antigen and acetylation of the sulfhydryl groups.

2. MATERIALS AND METHODS

2.1. *Peptide synthesis and production of anti-peptide antibodies*

A panel of 27 synthetic peptides were prepared using the Boc or Fmoc procedures [1,2]. They correspond to the following fragments of gp51: amino acids 21–27, 38–57, 39–48, 57–67, 59–69, 64–73, 68–87, 74–83, 78–92 (linear and cyclic), 81–90, 82–91, 84–103, 98–117, 104–123, 119–132, 124–143, 142–161, 144–155, 144–157, 168–180, 169–188, 195–205, 219–238, 249–268, 255–268, 260–268 (the amino acid numbering did not include the signal peptide; as reported in [3]).

These peptides were conjugated to keyhole limpet hemocyanin, emulsified with Freund adjuvant and injected via the intradermal route in rabbits. ELISA antibody titers were determined using as antigens 100 ng of peptides adsorbed onto the walls of the well. Titers are expressed as the inverse of the serum dilution for which the optical density was twice that of the negative control (preimmune serum)[2].

2.2. *Western blotting*

BLV particles were the sources of antigens. In some experiments, partially purified gp51 was also used [4].

The protein sample was boiled for 3 min in 400 ml of buffer (Tris-HCl 15 mM, pH 6.8; SDS 2%; 2-mercaptoethanol 3%; Bromophenol blue 0.005%; glycerol 10%). For the iodoacetamide treatment, the protein sample was boiled for 3 min in 300 ml of buffer (Tris-HCl 0.2 M, pH 8.3; SDS 1%; DTT 0.5%; EDTA 1 mM; Bromophenol blue 0.005%; glycerol 10%). After cooling, a 100 ml solution of iodoacetamide (15 mg of the reagent) was added and incubation was resumed for 1 h at room temperature in the dark. Immobilon (Millipore) strips on which electrophoretically fractionated BLV antigens (±0.5 mg of gp51 per strip) had been blotted were incubated for 30 min in phosphate buffer saline (PBS; sodium phosphate 0.01 M; NaCl 0.15 M, pH 7.4) containing 2% of bovine serum albumin to block non-specific sites. Strips were then incubated overnight at 4°C with anti-peptide antisera (1:30) or with purified anti-gp51 monoclonal antibodies [5] (1:100) in PBS containing 0.2% Tween 80. Strips were washed with PBS/Tween 80 0.2% and then with TBST buffer (Tris-HCl 0.01 M, pH 7.8; Tween 20 0.05%; NaCl 150 mM) before a 1 h incubation with goat anti-rabbit (for antisera) or goat anti-mouse (for monoclonal antibodies) immunoglobulins conjugated to alkaline phosphatase (1:100). WB reactivity was revealed, after extensive washings with TBST buffer, using a substrate containing 3.3 mg of NBT (Nitroblue tetrazolium) and 5 mg of BCIP (3-bromo-4-chloro-3-indolyl-phosphate (toluidine salt)) in 10 ml of AP buffer (Tris-HCl 10 mM, pH 9.5; NaCl 100 mM; $MgCl_2$ 5 mM).

3. RESULTS

ELISA antibody titers obtained with the 27 peptides mentioned above were reported in previous papers [1,2] and some are recalled in Table I for a few illustrative purposes. From Table I and Fig. 1, it is apparent that antibodies to peptides 142–161 and 255–268 gave a significant reaction in the classical WB test; antisera to

Abbreviations: BLV, bovine leukemia virus; ELISA, enzyme-linked immunosorbent assay; WB, Western blotting; RIA, radioimmunoassay; Mono A, monoclonal antibody against site A.

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 Published by Elsevier Science Publishers B.V.

Table I

Western blot analysis of gp51 antigen with anti-peptide antibodies

Rabbit number	peptide or MAb code	Amino acid sequence	anti-peptide antibody titer	Western blotting reactivity	
				without iodoacetamide treatment	with iodoacetamide treatment
14	82–91	PYVGADRFDC	1 090 000	–	+++
15	81–90	CPYVGADRFD	14 580	–	+
17	84–103	VGADRFDCPHWDNASQADQG	>3 300 000	–	+++
20	74–83	TYDCEPRCPY	3 300 000	–	–
21	169–188	LNQTARAFPDCAICWEPSPP	>3 300 000	–	–
99	168–180	LLNQTARAFPDCA	3 300 000	–	+++
92	142–161	KIPDPPQPDFPQLNSDWVPS	>9 900 000	++	+++
89	68–87	GARAMVTYDCEPRCPYVGAD	>9 900 000	+	+
93	219–238	NSSSFNTTQGWHHPSQRLLF	4 900 000	+	+
2664	255–268	STVSSAPPTRVRR	44 000	+++	+++
	MONO A	–	–	+++++	+++++

Note: +++++, strong reaction; +++, ++, good reaction; +, weak reaction; –, no reaction.

peptides 68–87 and 219–238 were only weakly positive. In contrast, iodoacetamide treatment revealed excellent WB reactivity for previously negative sera such as anti-peptides 82–91, 84–103, and 168–180. Anti-81–90 serum became slightly positive whilst the reaction encountered with anti 142–161 was definitely increased. By comparison, the anti-74–83 and 169–188 sera are illustrative of the behavior of the other 19 anti-peptide sera; they remained unreactive in WB of the entire gp51 even after acetylation of sulfhydryl groups. The reactivity of the anti-peptide antibodies, as observed after antigen treatment with iodoacetamide, cannot be considered as non-specific since all preimmune sera remained negative after iodoacetamide treatment, as shown on Fig. 1 for three of them (Rabbits 14, 92, 21, lines BI).

4. DISCUSSION

Previous studies [1,2] and more recent observations with new anti-peptide antibody systems indicated that only two regions of gp51 are well recognized by rabbit anti-peptide sera in classical WB. The first region is the carboxylic end of the protein (peptide 255–268)[1]; the second (142–161) corresponds to a putative hinge between the NH_2 and COOH moieties of gp51 [2]. Both regions seem to be particularly accessible even on the native envelope protein.

Among the 6 sera that are presented in Table I and that did not react in classical WB, 3 became strongly positive after iodoacetamide treatment, the 3 others either slightly improved (anti-81–90) or remained nega-

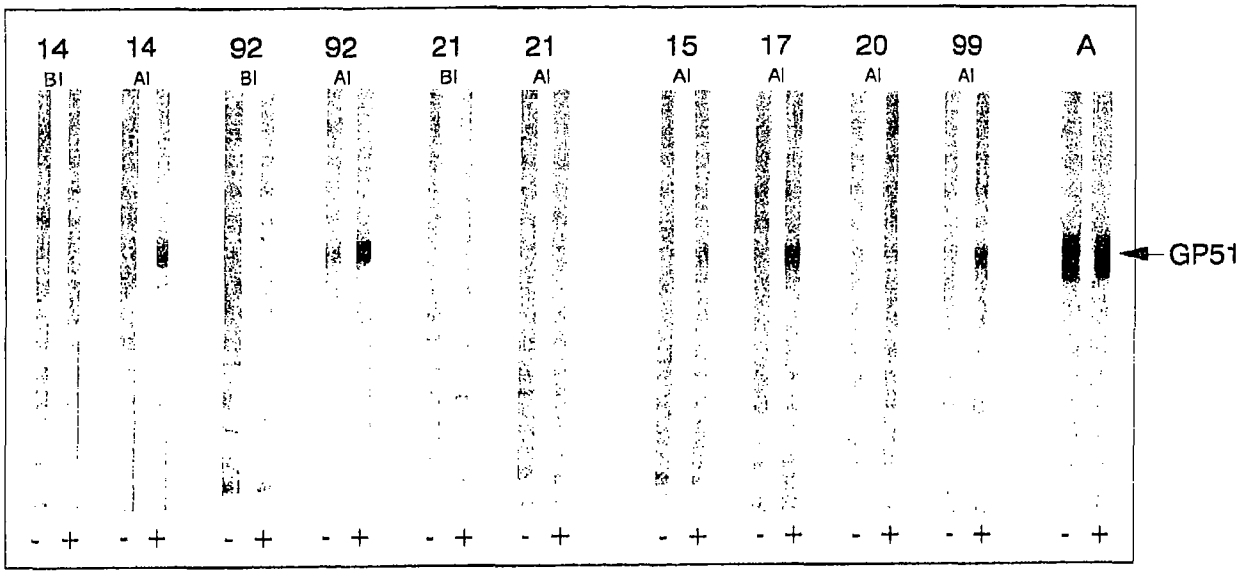

Fig. 1. Western blot analysis of BLV gp51 antigen with rabbit sera and monoclonal antibodies. Before running the gel BLV-gp51 was subjected to SDS-reducing agent denaturation followed (+) or not (–) by iodoacetamide treatment. The numbers at the top identify the rabbit antiserum; BI represents serum before immunization (preimmune serum), AI serum after 3 injections of peptide (anti-peptide antibodies); Mono A was used as a positive control.

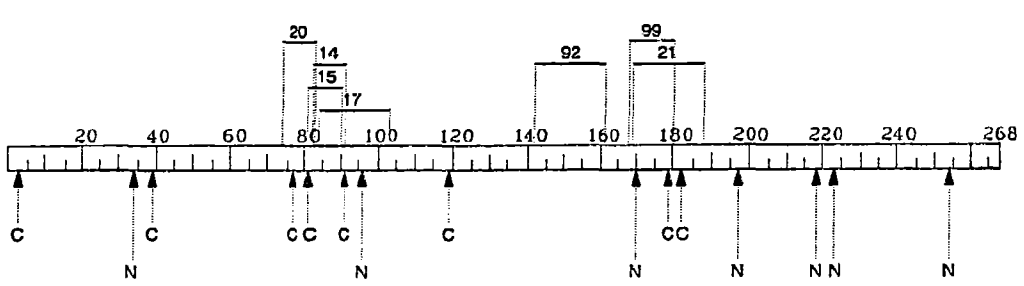

Fig. 2. Schematic representation of the gp51 polypeptide backbone. The positions of cysteine (C) residues, potential *N*-glycosylation sites (N) and selected anti-peptide antisera (identified by the rabbit number (14, 15 ...) are indicated.

tive (anti-74–83 and -169–188). It is of probable significance to note that in the 3 cases (anti-82–91, -84–103, -168–180) where a sharp change in antigen-antibody reaction was noticed, one C residue was present in the peptide sequence (Table I and Fig. 2). In the 2 cases where no reactivity was observed, whether acetylation was performed or not, a cysteine was also present in the peptide. Of course, acetylation of a C residue involved in a S–S bond will strongly contribute to the denaturation of the protein and make the corresponding region more amenable to react with anti-peptide antibody. In the cases where iodoacetamide treatment did not allow or increase recognition of the full size protein by the anti-peptide antibody, it can be surmised that the epitope recognized in the peptide encounters structural constraints in the protein. Sugar side chains are obvious candidates to account for the lack of antibody reactivity (Fig. 2) as they amount to 40% of the overall molecular weight of gp51 [4].

Such considerations are just speculations. They will be testable experimentally once the tridimensional organization of gp51 will be available.

In practical terms, we draw the investigators' attention to the fact that WB does not imply full denaturation of the protein and free access to any part of the protein backbone. Iodoacetamide treatment either keeps unchanged or significantly increases the reactivity of anti-peptide antibodies with the relevant region of the blotted protein. In no case did we experience a reduction of that reactivity; in all cases also the reactivity of preimmune rabbit sera remained negative after gp51 acetylation.

Acknowledgements: We thank Michèle Nuttinck, Guy Buchet and Michel Prevot for the preparation of the manuscript and figures. Isabelle Callebaut is Research Assistant of the Belgian National Fund for Scientific Research (FNRS). We are indebted to Prof. A. Tartar and H. Gras-Masse (Institut Pasteur de Lille, France) for learning peptide chemistry and Dr. V. Krchnak (Research Institute for Feed Supplements and Veterinary Drugs, Jilove ù Prahy, Czechoslovakia) and Dr. M. Merza (Biochemistry, Biomedical Center, Uppsala, Sweden) for providing some synthetic peptides.

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