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ANTI-PEPTIDE REAGENT IDENTIFIES A PRIMARY-STRUCTURE-DEPENDENT, CROSS-REACTIVE IDIOTYPE EXPRESSED ON HEAVY AND LIGHT CHAINS FROM A MURINE MONOCLONAL ANTI-CD4*

ROBERTA ATTANASIO,† PATRICK KANDA,† GREGORY W. STUNZ,† DAVID W. BUCK‡ and RONALD C. KENNEDY†§

†Department of Virology and Immunology and Center for AIDS Research, Southwest Foundation for Biomedical Research, San Antonio, TX 78228-0147, U.S.A.; and ‡Becton Dickinson Immunocytometry System, San Jose, CA 95131-1807, U.S.A.

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Abstract—A synthetic peptide corresponding to the second complementarity determining region (CDR2) of the immunoglobulin (Ig) variable (V) region heavy (H) chain ($CDR2V_H$) of anti-Leu3a, a murine monoclonal antibody specific for the human CD4 molecule, was used to elicit the production of specific rabbit anti-peptide antibodies. The rabbit anti-peptide antiserum was tested for reactivity against the immunizing peptide, anti-Leu3a, and a panel of mouse monoclonal anti-CD4. Only the immunizing peptide and anti-Leu3a were recognized by ELISA, whereas the H chains of anti-Leu3a and five other monoclonal anti-CD4 preparations were recognized by Western blot analysis. These data suggest that linear structures corresponding to the $CDR2V_H$ are not normally exposed on the surface of these monoclonal antibodies and become accessible only upon unfolding of the Ig molecule. In addition, Western blot analysis demonstrated that the anti-$CDR2V_H$ peptide antiserum was able to recognize the Ig light (L) chain of anti-Leu3a. This reactivity to both H and L chains from anti-Leu3a was ascribed to a homologous five amino acid sequence region shared by the two chains. The region of homology was associated with the third framework (FR3) of the L chain and was included as a portion of the sequence in the $CDR2V_H$ synthetic peptide. This observation was confirmed by the ability of the $CDR2V_H$ anti-peptide antiserum to bind the L chains of three mouse myeloma proteins that exhibited the five amino acid sequence region of homology within their respective FR3. Together, these data provide information on the structural basis of idiotypes shared by the H and L chains from the same antibody molecule and indicate that five amino acids might be sufficient to define a minimal continuous idiotypic determinant.

INTRODUCTION

Antigen recognition and idiotype (Id) expression represent the two aspects of the functional duality of the V region of the Ig molecule. The study of the structural correlates of these functions is of fundamental value for understanding the basic mechanisms involved in antigen–antibody recognition and the immunogenic properties of the Ig V region. The ability of the V region of an antibody molecule to express Id constitutes the basis of a network of interactions strictly involved in the regulation of the immune response (Jerne, 1974, 1984). This network is activated via the production of anti-idiotypic antibodies (anti-Id) directed to specific Id, which represent the intrinsic antigenic determinants of the immunoglobulin V region. The term idiotope defines a single V region antigenic determinant and, collectively, several idiotopes comprise the Id. An Id may be associated with the antigen-combining site of the antibody molecule or can be localized to regions not associated with the antigen-combining site. In addition, Id can be unique to a particular antibody molecule (private Id or IdI) or can be shared by several antibody molecules with the same or different specificity (cross-reactive Id or IdX) (reviewed in Eichman, 1978).

The structural correlates of idiotypy have been studied in a number of experimental antigen–antibody systems using a variety of strategies. These strategies include: (i)

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§Author to whom correspondence should be addressed at: Center for AIDS Research, Southwest Foundation for Biomedical Research, P.O. Box 28147, San Antonio, TX 78228-0147, U.S.A. Tel.: (512) 674-1410, Ext. 485/486; Fax: (512) 674-8549.

Abbreviations: anti-Id, anti-idiotypic antibody; BBS, borate buffered saline; CDR, complementarity determining region; $CDR2V_H$, second CDR of V_H; $CDR1V_\kappa$, first CDR of V_κ; $CDR3V_\kappa$, third CDR of V_κ; ELISA, enzyme-linked immunosorbent assay; FR3, third framework region; H, immunoglobulin heavy chain; HBsAg, hepatitis B virus surface antigen; HIV, human immunodeficiency virus; HRP-gargg, horseradish peroxidase conjugated goat anti-rabbit gammaglobulin; Id, idiotype; Ig, immunoglobulin; KLH, keyhole limpet hemocyanin; L, immunoglobulin light chain; NGS, normal goat serum; T-BBS, Tween-20 containing BBS; T-PBS, Tween-20 containing PBS; V_H, heavy chain variable region; V_L, light chain variable region; V_κ, kappa chain variable region.

Table 1. Summary of the mouse monoclonal antibodies utilized in this study

Antibody designation	Specificity	Isotype	Inhibition of CD4-anti-Leu3a binding	Recognized by anti-CDR2V_H antiserum by ELISA	Recognized by anti-CDR2V_H antiserum by Western blot (H chain specificity)	Recognized by anti-CDR2V_H antiserum by Western blot (L chain specificity)
Anti-Leu3a	CD4	IgG1/$_\kappa$	+[a]	+	+	+
L34	CD4	IgG1/$_\kappa$	+	−	−	−
L77	CD4	IgG1/$_\kappa$	+	−	−	−
L80	CD4	IgG1/$_\kappa$	+	−	+	−
L83	CD4	IgG1/$_\kappa$	−	−	−	−
L93	CD4	IgG1/$_\kappa$	+/−	−	−	−
L104	CD4	IgG2a/$_\kappa$	−	−	−	−
L110	CD4	IgG1/$_\kappa$	+	−	+	−
L112	CD4	IgG2b/$_\kappa$	−	−	−	−
L198	CD4	IgG1/$_\kappa$	+	−	−	−
L199	CD4	IgG2a/$_\kappa$	+	−	+	−
L200	CD4	IgG1/$_\kappa$	+	−	−	−
L202	CD4	IgG1/$_\kappa$	+	−	+	−
L204	CD4	IgG2a/$_\kappa$	+	−	−	−
L206	CD4	IgG1/$_\kappa$	+	−	−	−
L208	CD4	IgG1/$_\kappa$	+	−	+	−
A1.2	HBsAg	IgG1/$_\kappa$	−	−	−	−
A2.1	HBsAg	IgG1/$_\kappa$	−	−	−	−
MOPC 21	unknown	IgG1/$_\kappa$	−	−	−	+
MPC 11	unknown	IgG2b/$_\kappa$	−	−	−	+
TEPC 15	PC[b]	IgA/$_\kappa$	−	−	−	+

[a]Inhibition of binding was determined using fluorescein isothiocyanate conjugated anti-Leu3a binding to a CD4 expressing human T-cell line. (+) inhibition of binding greater than 90%; (+ / −) inhibition of binding between 40 and 60%; (−) inhibition less than 10%.

[b]Phosphorylcholine.

comparison of the amino acid sequences of Id positive and Id negative antibodies (Clevinger *et al.*, 1980; Rudikoff *et al.*, 1983; Bedzyk *et al.*, 1990); (ii) chemical modification experiments (Dickerman *et al.*, 1981); (iii) analysis of spontaneous V region variants (Radbruch *et al.*, 1985); (iv) site-directed mutagenesis (Sharon, 1990); and (v) use of synthetic peptides corresponding to the various CDRs of the Ig molecules (Chen *et al.*, 1984; McMillan *et al.*, 1983). The results of these studies have indicated that the expression of a given Id may be associated with isolated V region H (V_H) and V region L (V_L) chains of the antibody molecules. However, Id expression more frequently requires the association of both H and L chains. In addition, structural characterization of Id has identified the existence of both conformational and linear determinants and has correlated a particular Id with specific amino acid sequences within the CDRs of the antibody V region.

In this present study, we attempted to further characterize the structural correlates of the Id associated with anti-Leu3a, a mouse monoclonal antibody with specificity for the human CD4 molecule. This monoclonal antibody recognizes a CD4 epitope involved in the human immunodeficiency virus (HIV) gp120 binding and represents a focal point for understanding Id networks associated with the CD4–HIV gp120 interaction (reviewed in Attanasio and Kennedy, 1990). A synthetic peptide corresponding to the second CDR of the H chain (CDR2V_H) of anti-Leu3a was used to generate antibodies specific for both the peptide and the cognate anti-Leu3a molecule. The results of this study indicate that epitopes associated with conformationally unrestricted structures corresponding to the CDR2V_H of various monoclonal anti-CD4 are not exposed on the surface of the molecule but become accessible upon its unfolding. In addition, a primary-structure-dependent, cross-reactive Id expressed on both isolated H and L chains of anti-Leu3a was structurally correlated to the presence of a five amino acid sequence region of homology. This sequence homology was observed in the third framework region (FR3) of the anti-Leu3a L chain and represents part of the CDR2V_H peptide sequence used to generate the anti-peptide reagents. Studies utilizing mouse myeloma proteins whose V region sequences are known indicated that five contiguous amino acids may define the minimal sequence requirements for Id expression.

MATERIALS AND METHODS

Monoclonal antibodies and myeloma proteins

The murine monoclonal anti-CD4 preparations used in this study and their CD4 epitope recognition and ability to inhibit HIV infectivity *in vitro* have been described in detail elsewhere (Attanasio *et al.*, 1991*a*). To determine CD4 epitope recognition, the various monoclonal antibodies used as inhibitors (5 μg each) were examined by flow cytometry for their ability to

inhibit anti-Leu3a conjugated with fluorescein isothiocyanate from binding to the human CD4 positive T-cell line, HPB-ALL. Anti-Leu3a recognizes a CD4 epitope associated with the HIV-1 gp120 binding site (Attanasio *et al.*, 1991*a*). The murine myeloma protein MOPC 21 was obtained from Sigma Chemical Co. (St Louis, MO). The murine myeloma proteins MPC 11 and TEPC 15 were kindly provided by Dr C. A. Bona. The irrelevant mouse monoclonal antibodies used in this study, designated A1.2 and A2.1, exhibit specificity for hepatitis B virus surface antigen (HBsAg). The antigenic and isotypic specificities of all the mouse monoclonal antibodies and myeloma proteins utilized in this study are summarized in Table 1.

V region sequences

The V region, kappa chain (V_κ) amino acid sequence for anti-Leu3a has been described elsewhere (Attanasio *et al.*, 1991*b*). The cDNA and deduced amino acid sequence of the V_κ region of murine monoclonal anti-CD4 preparations L34, L77, L93, and L202 were determined by methods previously described (Attanasio *et al.*, 1991*b*). Anti-Leu3a V_H region amino acid sequence was determined as previously described (Lohman *et al.*, 1991). The V region amino acid sequences of mouse myeloma proteins MPC 11, MOPC 21, and TEPC 15 have been described elsewhere (Kabat *et al.*, 1987).

Peptide synthesis

A synthetic peptide representing the anti-Leu3a H chain V region sequence 49–66 was assembled by solid phase methodology. The synthesis was initiated with a glycine linked to the polystyrene resin via the 2-(4′-hydroxymethyl-phenoxy)-acetyl linker. The synthesis chemistry was based on Fmoc alpha-amino protection, and the peptide was made using a Biosearch 9500 automated synthesizer. Side chain protection was as follows: tert-butyl ester for ASP and GLU; tert-butyl ethers for the SER, THR, and TYR hydroxyls; tert-butyl-thiol for the CYS sulfhydryl; and tert-butyl-oxycarbonyl for the LYS epsilon-amino groups. Following synthesis, the peptide was cleaved from the resin with simultaneous deprotection of the amino acid side chains using a mixture of TFA:water (95:5, 10 ml/g resin). The crude peptide was desalted on a Biogel P-2 column, followed by HPLC purification on a VyDac C8 reversed phase column. The purity was assessed by amino acid analysis of the HPLC purified material. The sequence of the anti-Leu3a $CDR2V_H$ synthetic peptide was CYS–GLY–GLU–THR–TYR–THR–GLY–SER–GLY–SER–SER–TYR–TYR–ASN–GLU–LYS–PHE–LYS–ASP–GLY. The CYS was added to the amino terminus to facilitate coupling to carrier proteins for immunization of rabbits. Synthetic peptides corresponding to the first ($CDR1V_\kappa$) and third ($CDR3V_\kappa$) CDR of the L chain of anti-Leu3a were used as controls and have been described in detail elsewhere (Attanasio *et al.*, 1991*b*).

Production of anti-peptide antibodies

Two New Zealand white female rabbits were injected intramuscularly with 200 μg of $CDR2V_H$ synthetic peptide coupled to keyhole limpet hemocyanin (KLH). The injections were administered at monthly intervals. For the initial injection, the synthetic peptide was emulsified in Freund's complete adjuvant, and for subsequent immunizations in Freund's incomplete adjuvant. Rabbits received a total of six injections. Serum obtained from each animal prior to the first injection served as a control. The animals were bled every 2 weeks following the third immunization. Sera obtained after the sixth injection were tested by ELISA for reactivity with the immunizing peptide along with irrelevant peptides. Both rabbits produced specific anti-peptide antibodies (data not shown). One antiserum was selected to perform all of the described studies following adsorption over an Affi-gel 10 column (Bio-Rad Laboratories, Richmond, CA) containing a pool of normal mouse Ig. This adsorption was performed to eliminate all nonspecific reactivity to murine Ig. No difference in the antibody endpoint titers to either the $CDR2V_H$ synthetic peptide or anti-Leu3a was observed by using unadsorbed or adsorbed serum.

KLH coupling

The $CDR2V_H$ synthetic peptide was coupled to KLH (Calbiochem Co., San Diego, CA) by using the heterobifunctional crosslinker *m*-maleimidobenzoyl-*N*-hydroxy-sulfosuccinimide ester. The KLH was activated with a 300-fold molar excess of SMBS for 1 hr at room temp in 0.05 M borate-buffered saline (BBS), pH 7.5, then dialyzed against BBS. The peptide was treated with a 10-fold molar excess of dithiothreitol in nitrogen-purged BBS, pH 8.0, to reduce the cysteine sulfhydryl, and the excess dithiothreitol was removed by passage of the mixture through a Biogel P-2 column. The peptide was added to the activated KLH in BBS, pH 8.0, and the mixture was stirred for 2 hr at room temp. The uncoupled peptide was removed by dialysis against several changes of PBS, pH 7.5, at 4°C. The molar ratio of peptide to KLH in the conjugate was determined by amino acid analysis (Briand *et al.*, 1985).

Direct binding assays

The characterization of the anti-$CDR2V_H$ antiserum for the ability to recognize the $CDR2V_H$ synthetic peptide and anti-Leu3a, along with control CDR synthetic peptides and other mouse monoclonal antibodies, was assessed by ELISA. Briefly, 200 ng of the various monoclonal antibodies or synthetic peptides was diluted in 50 μl of BBS, added to individual wells of 96-well polystyrene microtiter plates (Corning Glass Works, Corning, NY) and allowed to adsorb to the solid phase overnight at 4°C. After blocking of non-specific binding sites with 10% normal goat serum (NGS) for 30 min at 37°C, the plate was washed with 0.01% Tween-20 PBS (T-PBS). Serial dilutions of rabbit antiserum in 10%

NGS were added to triplicate wells for 2 hr at 37°C, and the plate was washed with T-PBS. Horseradish peroxidase conjugated goat anti-rabbit gammaglobulin (HRP-gargg; KPL, Gaithersburg, MD) was diluted in 10% NGS and added for 1 hr at 37°C. The plate was then washed with T-PBS. The substrate was 15 mg/ml of 2,2′-azino-di-(3-ethyl-benzthiazoline sulfonic acid) (ABTS; Sigma Chemical Co., St Louis, MO) containing 0.01% H_2O_2. The addition of 5% SDS was used to terminate the reaction. Optical density values for each well were determined on an automatic ELISA plate reader (MR600; Dynatech Laboratories, Inc., Alexandria, VA) at 410 nm (OD_{410}). Background absorbance levels, obtained by using each of the reagents except for the rabbit antiserum, were subtracted from each microtiter well. The determination of binding was based on an OD_{410} value three times greater than the value obtained with irrelevant mouse monoclonal antibodies and irrelevant synthetic peptides at a reciprocal dilution of 100.

Competitive inhibition assays

The ability of the free synthetic $CDR2V_H$ peptide to inhibit the binding of the rabbit anti-peptide antiserum to anti-Leu3a was examined using a competitive inhibition ELISA. Two hundred nanograms of anti-Leu3a in 50 μl of BBS was coated onto microtiter wells overnight at 4°C. Nonspecific binding sites were blocked by adding 10% NGS for 30 min at 37°C, and then the plate was washed in T-PBS. A dilution in 10% NGS of rabbit antisera corresponding to the linear part of the anti-Leu3a binding curve was mixed with different concentrations of the free peptides, added to triplicate wells in 50 μl aliquots, and allowed to incubate overnight at 4°C. After washing with T-PBS, HRP-gargg was added to each well for 1 hr at 37°C. The addition of the substrate was the same as described for the direct binding ELISA.

The percent inhibition was calculated with the formula:

$$\% \text{ inhibition} = 100 \times \left[1 - \frac{(OD_{410} \text{ with inhibitor} - \text{background})}{(OD_{410} \text{ without inhibitor} - \text{background})}\right].$$

Western blot analysis

The H and L chain specificity of the rabbit anti-peptide antisera was determined by Western blot analysis. Heavy and L chains of the various mouse monoclonal anti-CD4 antibodies, along with irrelevant mouse monoclonal antibody preparations, were denatured and separated by discontinuous SDS–PAGE according to Laemmli (1970). Proteins were electrophoretically transferred to nitrocellulose membrane at 80 V for 4 hr. After transfer, non-specific binding sites on the membrane were blocked with Blotto (2.5% non-fat dry milk, 2.5% liquid gelatin, 0.05% Tween-20, 0.01% thimerosal, 0.001% antifoam A) overnight at 4°C. The nitrocellulose membrane was washed with 0.05% Tween-20 BBS (T-BBS) and incubated with a 1:50 dilution of rabbit antiserum overnight at 4°C. The membrane was washed with T-BBS, incubated with ^{125}I-Protein A (DuPont, Wilmington, DE) in Blotto for 2 hr at room temp, and again washed extensively with T-BBS, dried, and exposed to X-ray film overnight at −70°C. To ascertain whether H and L chains of the monoclonal antibodies had been transferred to the nitrocellulose, the paper was incubated again with a rabbit antiserum specific for mouse IgG. The membrane was then incubated with ^{125}I-Protein A, dried, and exposed to X-ray film.

The ability of the free $CDR2V_H$ synthetic peptide to inhibit the binding of the rabbit anti-peptide antiserum to both H and L chains of various monoclonal antibodies was determined by using Western blot analysis similar to that described above, with the following modification. Rabbit antiserum (diluted 1:50) in Blotto was mixed with either a 10 μg/ml concn of $CDR2V_H$ peptide or a similar concn of a control peptide (anti-Leu3a $CDR1V_\kappa$) and incubated with the nitrocellulose membrane overnight at 4°C. The remainder of the assay was performed as described above.

RESULTS

Rabbit anti-$CDR2V_H$ peptide antiserum recognizes both the immunizing peptide and the cognate anti-Leu3a molecule

To determine whether the anti-Leu3a $CDR2V_H$ elicited the production of anti-peptide antibodies that recognized both the immunizing peptide and the whole anti-Leu3a molecule, a direct binding ELISA was performed. The $CDR2V_H$ synthetic peptide and anti-Leu3a, along with irrelevant CDR synthetic peptides and mouse monoclonal antibodies, were bound to the solid phase. Serial dilutions of the rabbit antiserum were added to the microtiter wells. As shown in Fig. 1, the rabbit anti-peptide antiserum bound both the $CDR2V_H$ peptide and the whole anti-Leu3a molecule. At a reciprocal dilution of 100, an OD_{410} value greater than 1.0 was observed with the $CDR2V_H$ peptide and anti-Leu3a, whereas an OD value less than 0.1 was observed with irrelevant peptides and irrelevant monoclonal antibodies. Serum obtained prior to immunization failed to bind either the $CDR2V_H$ peptide or anti-Leu3a (data not shown). These results demonstrated that antibodies induced by immunizing with the $CDR2V_H$ synthetic peptides were able to recognize the cognate antibody molecule. To confirm that the anti-Leu3a reactivity was due to the recognition of an amino acid sequence corresponding to the $CDR2V_H$ synthetic peptide, a competitive inhibition ELISA was performed. Various concns of the free $CDR2V_H$ peptide, along with irrelevant synthetic peptides, were used to inhibit the binding of the anti-peptide antiserum to anti-Leu3a. The resulting inhibition curves are shown in Fig. 2. At a concn of 1000 ng/ml, the free $CDR2V_H$ peptide inhibited the anti-Leu3a–anti-peptide reaction by greater than 80%; no inhibition was observed using similar concentrations of irrelevant peptides. These results suggest that the

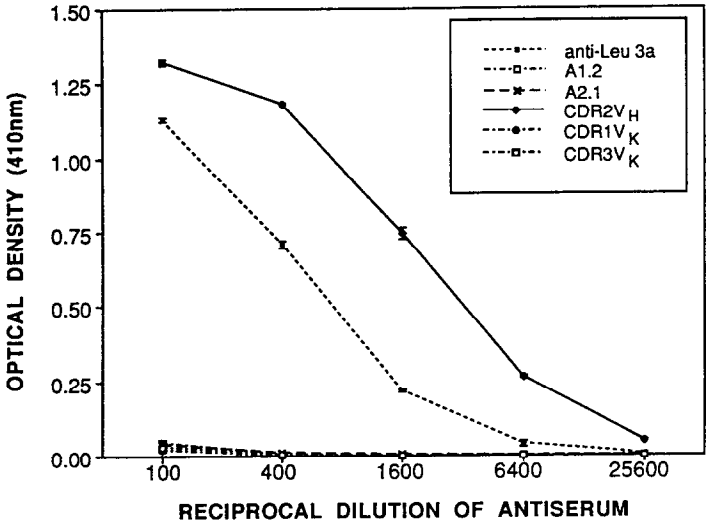

Fig. 1. Binding curves of anti-CDR2V_H peptide antiserum to synthetic peptides and mouse monoclonal antibodies. Each point represents the mean of triplicate determinations. The vertical bars represent ± 1 standard deviation from the mean value. CDR1V_κ and CDR3V_κ represent irrelevant peptides. A1.2 and A2.1 represent irrelevant mouse monoclonal antibodies.

ability of the anti-CDR2V_H peptide antiserum to bind anti-Leu3a is due to the recognition of the CDR2V_H amino acid sequence.

In the next set of experiments, we assessed whether the anti-CDR2V_H peptide antiserum was able to bind other monoclonal anti-CD4 by ELISA. Microtiter wells were coated with the various monoclonal anti-CD4 antibodies, and serial dilutions of the rabbit anti-CDR2V_H peptide antiserum were added. No significant reactivity was observed with any of the other monoclonal anti-CD4 tested. These results are summarized in Table 1.

Rabbit anti-CDR2V_H peptide antiserum recognizes H and L chains of anti-Leu3a along with the H chains of several other mouse monoclonal anti-CD4

To confirm the binding characteristics observed by ELISA along with the anti-Leu3a H chain specificity of the anti-CDR2V_H peptide antiserum, we examined the various mouse monoclonal antibodies for reactivity with the anti-peptide antiserum by Western blot analysis. We also characterized CD4 epitope recognition based on the ability of the monoclonal anti-CD4 to inhibit anti-Leu3a from binding to cell surface expressed CD4 (Table 1). Surprisingly, the anti-CDR2V_H peptide antiserum bound both H and L chains of anti-Leu3a. In addition, the rabbit anti-peptide antiserum bound the H chains of five other mouse anti-CD4 antibodies, designated L80, L110, L199, L202, and L208 [Fig. 3, panel (A)] that were not detected by ELISA (Table 1). Serum from a rabbit immunized with the native anti-Leu3a molecule, which was not adsorbed to remove isotypic specificities and was used as a positive control, detected both H and L chains of the various monoclonal antibodies [Fig. 3, panel (B)]. The reactivity of the anti-peptide antiserum

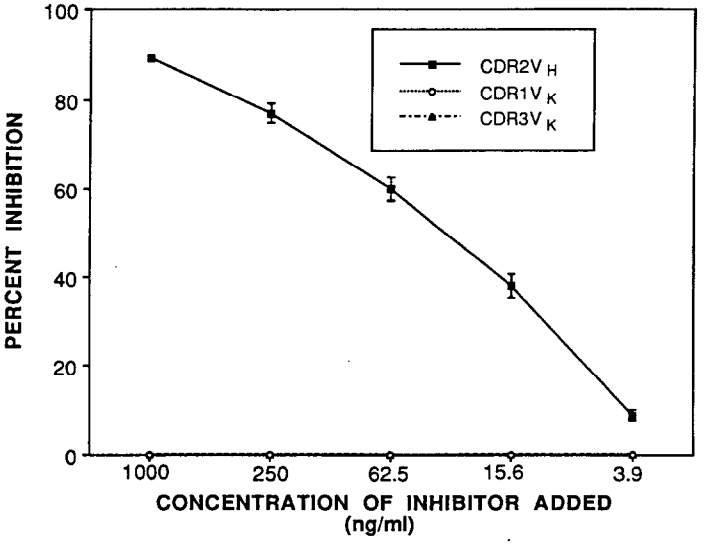

Fig. 2. Inhibition curves of rabbit anti-CDR2V_H peptide antiserum binding to anti-Leu3a using free synthetic peptides. Each point represent the mean of triplicate determinations. The vertical bars represent ± 1 standard deviation from the mean value.

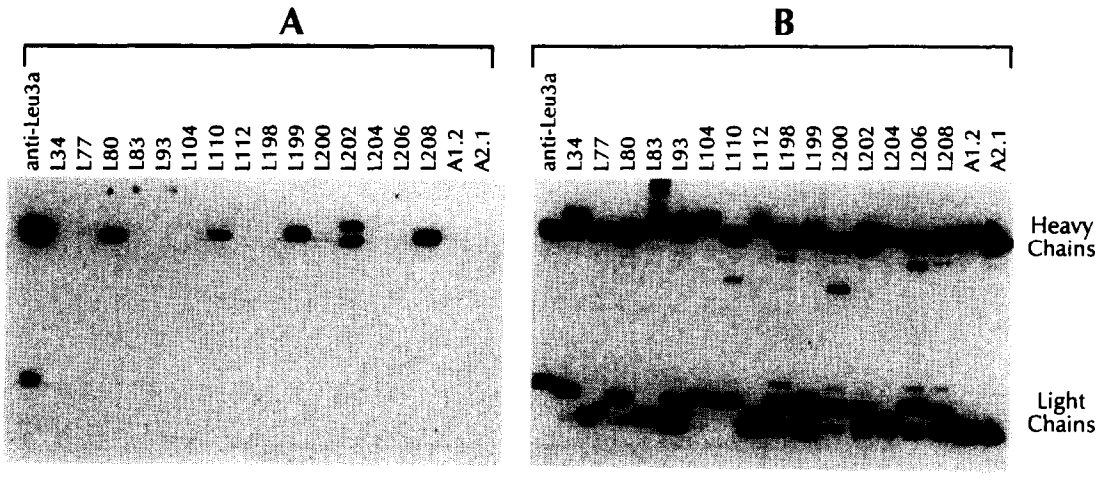

Fig. 3. Western blot analysis showing the binding of the rabbit anti-CDR2V_H peptide antiserum to isolated H and L chains of mouse monoclonal anti-CD4. (A) nitrocellulose membrane incubated with the rabbit anti-CDR2V_H peptide antiserum; (B) nitrocellulose membrane incubated with rabbit antiserum specific for mouse IgG.

to the various monoclonal antibodies obtained by Western blot analysis is summarized in Table I. Rabbit serum obtained prior to immunization with the anti-Leu3a CDR2V_H synthetic peptide failed to bind both H and L chains of the various monoclonal antibodies (data not shown). The inability of the anti-CDR2V_H peptide antiserum to bind some of the monoclonal anti-CD4, along with two irrelevant mouse monoclonal preparations indicated that both the H and L chain reactivities are specific. Together, these results suggest that a cross-reactive primary-structure-dependent Id determinant is shared by the H and L chains of anti-Leu3a. In addition, a primary-structure-dependent Id determinant that is detected by Western blot analysis but not by ELISA is also shared by the H chains of anti-Leu3a, L80, L110, L199, L202, and L208. The expression of this Id on anti-CD4 H chains is not associated directly with CD4 epitope recognition.

A five amino acid region of homology is involved in the expression of a primary-structure-dependent, cross-reactive Id shared by the H and L chains of anti-Leu3a

To assess whether a region of amino acid sequence homology was present in both H and L chains of anti-Leu3a, we compared the amino acid sequence of the CDR2V_H synthetic peptide with that of the L chain. A common sequence of five amino acids was identified at position 53–57 of the H chain and at position 67–71 (FR 3) of the L chain. These sequence similarities are depicted in Fig. 4.

Based on this primary sequence homology, we decided to attempt to confirm that the H and L chain cross-reactivity was due to the ability of the CDR2V_H peptide antiserum to recognize the five amino acid sequence homology region. For this purpose, Western blot analysis was performed using three mouse myeloma proteins (MPC 11, MOPC 21, and TEPC 15) which share with anti-Leu3a the presence of the five amino acids in the FR3 of the L chain. The rabbit anti-peptide antiserum was able to bind the L chains of anti-Leu3a, MPC 11, MOPC 21, and TEPC 15, whereas no binding was observed with the L chains of L34, L77, L93, and L202 [Fig. 5, panel (A)]. The L chains of these latter monoclonal anti-CD4 possess only four of the five amino acids within the sequence common to anti-Leu3a and the three mouse myeloma proteins with the THR at position 67 being substituted either by SER (L34, L77, L93) or glycine (L202).

To confirm that the reactivity observed by Western blot analysis was due to the recognition of amino acid structures associated with the CDR2V_H of anti-Leu3a, free CDR2V_H peptide was used to inhibit the binding of the rabbit anti-peptide antiserum to the various mouse monoclonal anti-CD4 and myeloma proteins. The CDR2V_H peptide was able to inhibit the binding of the rabbit anti-peptide antiserum to the H and L chains of the various monoclonal anti-CD4 preparations and mouse myeloma proteins [Fig. 5, panel (B)], whereas no significant inhibition was observed by using an irrelevant peptide [Fig. 5, panel (C)]. Together these data indicate that five amino acids may define the minimal sequence

50
CYS-GLY-GLU-THR-TYR-THR-GLY-SER-GLY-SER-SER-TYR-

66
TYR-ASN-GLU-LYS-PHE-LYS-ASP-GLY

61
GLY-ILE-PRO-ALA-ARG-PHE-THR-GLY-SER-GLY-SER-GLY-

80
THR-ASP-PHE-THR-LEU-ASN-ILE-HIS

Fig. 4. Comparison of the amino acid sequences of the anti-Leu3a CDR2V_H peptide (top) and a portion of the third framework region of the L chain of anti-Leu3a (bottom). The five amino acid region of homology is underlined in both sequences. The numbers represent the amino acid position within the H and L chains, respectively. In the CDR2V_H synthetic peptide, Gly49 is derived from the adjacent second framework.

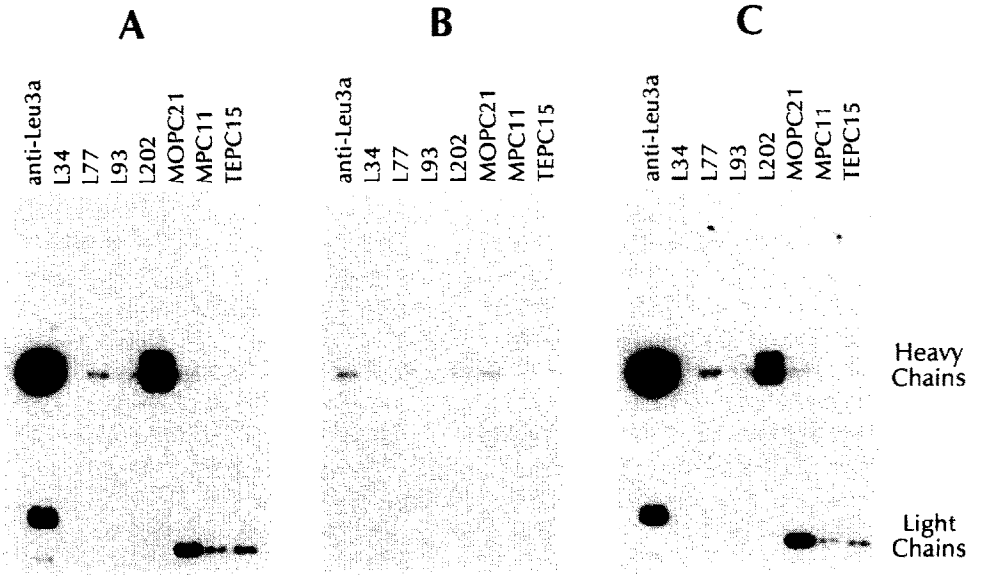

Fig. 5. Western blot analysis showing the binding of the rabbit anti-CDR2V_H peptide antiserum to various mouse monoclonal antibodies: (A) in absence of synthetic peptide; (B) in presence of CDR2V_H synthetic peptide; (C) in presence of CDR1V_κ synthetic peptide (irrelevant peptide).

requirement for Id expression in the murine monoclonal anti-CD4 system.

DISCUSSION

We initially attempted to determine whether a previously described private Id expressed on the H chain of anti-Leu3a (Attanasio *et al.*, 1991a) was associated with the CDR2V_H. A synthetic peptide corresponding to this region of anti-Leu3a was used to elicit the production of specific antibodies able to recognize both the synthetic peptide and the cognate anti-Leu3a molecule. The ability of the CDR2V_H peptide to inhibit the anti-Leu3a–anti-peptide antibody reaction indicated that the anti-Leu3a reactivity is due to the recognition of amino acid sequences corresponding to the CDR2V_H. The antipeptide antisera were able to recognize only anti-Leu3a by ELISA, whereas the H chain of anti-Leu3a along with the H chains of several other mouse monoclonal anti-CD4 were recognized by Western blot analysis. These data possibly indicate that linear structures corresponding to the CDR2V_H may not be exposed in the intact antibody molecules. The possibility exists that the unfolding of the Ig molecule following the reduction and denaturation process leads to the exposure of the CDR2V_H. Alternatively, it might be possible that either adsorption of the Ig molecules to the ELISA plates causes conformational changes that result in the inaccessibility of the epitope(s) corresponding to the CDR2V_H or that antigenic motifs recognized by the anti-peptide antiserum on different antibodies are different. Previous studies indicated that no binding to the H chain of monoclonal anti-CD4 other than anti-Leu3a is observed by Western blot analysis using anti-Id generated by immunizing with the native anti-Leu3a molecule (Attanasio *et al.*, 1991*a*). Our present findings seem to indicate that the response to the CDR2V_H synthetic peptide does not reflect the response to the native anti-Leu3a molecule. This is confirmed by the inability of anti-Id raised by immunizing with the intact anti-Leu3a molecule to bind the CDR2V_H synthetic peptide (data not shown). Together, these data indicate that the private Id expressed on the H chain of anti-Leu3a is not associated with linear motifs of the CDR2V_H.

An interesting observation emerging from this study is the ability of the anti-CDR2V_H peptide antisera to bind both isolated H and L chains of anti-Leu3a. Since the binding to the L chain appeared to be specific, the amino acid sequences from both H and L chains were compared to identify the presence of possible shared nucleotide and amino acid sequences. A stretch of five amino acids localized at positions 53–57 of the H chain of anti-Leu3a and included in the sequence of the CDR2V_H synthetic peptide was also present at position 67–71 within the FR3 of the L chain of anti-Leu3a. There appeared to be no direct association between the five amino acid region of sequence homology within the CDR2V_H and the expression of a particular V_H gene family. All monoclonal anti-CD4 CDR2V_H regions that expressed this sequence were encoded by V_H J558; however, other antibodies which did not possess this sequence were also encoded by members of the V_H J558 gene family. This region of amino acid sequence homology appears to be a fortuitous event, since homology exists at the nucleotide level for only two of the five amino acids. To verify whether the cross-reactivity observed between the H and L chains of anti-Leu3a was due to the presence of this five amino acid shared sequence, Western blot analysis was performed to assess the ability of the anti-CDR2V_H peptide antiserum to bind the L chain of anti-Leu3a along with the L chains of four additional mouse monoclonal anti-CD4 preparations and three mouse myeloma proteins of known amino acid sequence. This five amino acid sequence within the FR3 of murine L

chains was associated with a $V_\kappa 21$ subgroup encoded by the $V_\kappa 21$ germline gene family. However, other monoclonal anti-CD4 preparations that expressed similar $V_\kappa 21$ encoded L chains and were not recognized by the anti-peptide antiserum exhibited a GLY or SER substitution for THR at position 67 in the FR3V of anti-Leu3a. Results from this experiment indicated that the reactivity observed with isolated H and L chains of anti-Leu3a might be due to the presence of the shared five amino acid sequence, since no binding was observed with the L chains of the four additional monoclonal anti-CD4. These four monoclonal anti-CD4 share sequence homology with the L chain of anti-Leu3a in only four of the five amino acids in the FR3. Conversely, the anti-$CDR2V_H$ peptide antiserum was able to bind the L chains of the three mouse myeloma proteins, which share with anti-Leu3a the stretch of five amino acids. The $CDR2V_H$ peptide was able to inhibit the binding of the anti-peptide antiserum to H and L chains of the various mouse preparations, confirming that the reactivity observed by Western blot analysis was due to the recognition of a structure associated with the anti-Leu3a $CDR2V_H$. To exclude the possibility that the cross-reactivity observed with anti-Leu3a and the three myeloma proteins was due to the presence of amino acid homologies different from those described above, the sequence of the $CDR2V_H$ synthetic peptide was compared with that of the L chains of the three myeloma proteins. No additional homology was observed, confirming that the ability of the anti-$CDR2V_H$ peptide antiserum to bind the L chains of the three mouse myeloma proteins might be due to the recognition of the stretch of five amino acids. No reactivity of the anti-$CDR2V_H$ peptide antiserum with the three mouse myeloma proteins was observed by ELISA (Table 1). This finding indicates that either the five amino acid epitope is hidden in the molecule, or only a small population of antibodies directed to the $CDR2V_H$ peptide is able to recognize the shared five amino acid sequence. In the latter case, the sensitivity of the ELISA might not be sufficient to detect the presence of this antibody subpopulation. Anti-Id obtained by immunizing with the native anti-Leu3a molecule were unable to bind the L chains of these three mouse myeloma proteins by ELISA and by Western blot analysis (data not shown), indicating that production of antibodies directed to the five amino acid sequence does not occur in detectable amounts during the normal response to the native anti-Leu3a molecule. Based on these data, it is difficult to evaluate the role that the five amino acid epitope plays during the idiotypic response directed to the intact anti-Leu3a molecule. However, this sequence is present on an antibody molecule V region and therefore, by definition, can be considered an idiotope. Our data indicate that the length of a primary structure-dependent idiotope might be represented by five amino acids, since the anti-$CDR2V_H$ peptide antiserum was unable to bind the L chain of mouse monoclonal antibodies in which the THR at position 67 of the L chain was substituted by a different amino acid. The possible role that the general conformation of the Ig molecule may play in the expression of the cross-reactive Id should also be considered. Cross-reactive Id independently expressed on both H and L chains of the immunoglobulin molecules have been described for a human myeloma protein (Matsuoka *et al.*, 1971), a human IgM cold agglutinin (Kobzik *et al.*, 1976), for a mouse monoclonal auto-antibody reacting with thyroglobulin (Zanetti and Rogers, 1987), and on mouse monoclonal anti-CD4 (Perosa *et al.*, 1991). The cross-reactive Id expressed on the anti-thyroglobulin monoclonal antibody is a regulatory idiotype. The simultaneous presence of this same Id on the H and L chains may confer regulatory properties to this antibody within the idiotypic network (Zanetti *et al.*, 1985). An Id shared by the H and L chains of the same antibody may therefore represent the basis of important functional properties. With the monoclonal anti-CD4 system, a monoclonal anti-Id was demonstrated to recognize isolated and denaturated H and L chains on an anti-CD4 preparation, designated HP2/6. These authors (Zanetti *et al.*, 1985; Perosa *et al.*, 1991) suggested that the recognition of this Id on both H and L chains was likely to be sequence-dependent. It has been suggested that the structural correlates of cross-reactive Id shared by the H and L chains of the Ig molecules are represented by the presence of shared sequences of amino acids (Kobzik *et al.*, 1976). Our data provide experimental evidence for this hypothesis and extend previous studies that implicate primary amino acid sequence homology as the basis for the expression of a cross-reactive idiotype on H and L chains from the same antibody molecule.

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