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Immunology

Induction of antibodies to the envelope protein of the human immunodeficiency virus by immunization with monoclonal anti-idiotypes

(internal image/mimicry/synthetic peptide)

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ABSTRACT **Anti-idiotypes that possess the internal image of antigen can induce protective humoral immunity toward microbes. Herein we demonstrate antigen mimicry by monoclonal anti-idiotypes of a distinct epitope of the human immunodeficiency virus (HIV) envelope protein that is defined by a synthetic peptide. This peptide, corresponding to amino acid residues 503–535 (peptide 503–535) of HIV-1 IIIB gp160, induced antibodies in three mammalian species that interacted with HIV-1 gp120 and inhibited *in vitro* syncytium formation caused by HIV-1, IIIB and MN isolates. Three monoclonal anti-idiotypes were generated against rabbit anti-gp120 antibodies specific for peptide 503–535. These anti-idiotypes recognize an interspecies cross-reactive idiotype expressed on mouse, chimpanzee, baboon, rabbit, and human anti-gp120 antibodies specific for peptide 503–535. The interaction with the cross-reactive idiotype is inhibited by synthetic peptide and HIV-1 gp160. Furthermore, rabbits immunized with the monoclonal anti-idiotypes produced antibodies that also bind HIV-1 gp120 and gp160 and recognized the epitope defined by peptide 503–535.**

A large body of evidence indicates that the human immunodeficiency virus (HIV) binds and infects the CD4-positive T cells through the envelope gene products. In addition, the envelope glycoprotein of HIV-1, gp160, bears epitopes capable of inducing neutralizing antibodies and cell-mediated immunity (1, 2). Of particular interest is peptide 503–535. This region encompasses an immunodominant epitope defined by peptide 504–518 (3) and a putative fusogenic site defined by peptide 526–535 (4). There are numerous approaches for developing safe and efficient vaccine against HIV (5). Two approaches that target immune responses to defined epitopes utilize either synthetic peptide-based technology or anti-idiotypes (anti-Ids) carrying the internal image of selected HIV antigenic determinants.

There are numerous observations indicating that anti-Ids can elicit specific immunity toward infectious agents (reviewed in ref. 6). Based on these considerations, our aim was to generate monoclonal anti-Ids to antibodies specific for selected epitopes expressed on the HIV-1 gp160 and to investigate the humoral immune response that would be induced subsequent to immunization with anti-Ids. Therefore, we have prepared in three mammalian species antibodies to a HIV-1 gp160 epitope defined by a synthetic peptide corresponding to amino acid residues 503–535 from human T-lymphotropic virus IIIB (HTLV IIIB) isolate of HIV-1. Rabbit anti-gp120 antibodies that recognized peptide 503–535 were used to generate anti-Id monoclonal antibodies (mAbs). Among a panel of nine anti-Id mAbs, three antibodies recognize an antigen-inhibitable interspecies cross-reactive Id (ISCRI). Furthermore, these anti-Ids induced antibodies specific for HIV-1 gp120 and gp160 epitopes defined by peptide 503–535 in rabbits.

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MATERIALS AND METHODS

Antigens. Synthetic peptides corresponding to amino acid residues 254–274 (Cys-Thr-His-Gly-Ile-Arg-Pro-Val-Val-Ser-Thr-Gln-Leu-Leu-Leu-Asn-Gly-Ser-Leu-Ala-Glu), 420–445 (Gly-Ile-Thr-Leu-Pro-Cys-Arg-Ile-Lys-Gln-Ile-Ile-Asn-Met-Trp-Gln-Glu-Val-Gly-Lys-Ala-Met-Tyr-Ala-Pro-Pro), 503–535-Gly-Cys (Val-Ala-Pro-Thr-Lys-Ala-Lys-Arg-Arg-Val-Val-Gln-Arg-Glu-Lys-Arg-Ala-Val-Gly-Ile-Gly-Ala-Leu-Phe-Leu-Gly-Phe-Leu-Gly-Ala-Ala-Gly-Ser-Gly-Cys), and Lys-Lys-Lys-526–535-Gly-Cys (Lys-Lys-Lys-Phe-Leu-Gly-Phe-Leu-Gly-Ala-Ala-Gly-Ser-Gly-Cys) from the gp160 envelope protein of HTLV IIIB isolate were synthesized. To the peptide sequence not containing a cysteine residue, a Gly-Cys dipeptide was added at the carboxyl terminus to provide a functional -SH group for coupling of the peptide to a carrier protein. In the case of peptide 526–535 three lysine residues were added at the amino terminus to improve peptide solubility. The peptides were synthesized on a *p*-methylbenzhydrylamine resin, purified on HPLC column, and coupled to either keyhole limpet hemocyanin (KLH) or bovine serum albumin (BSA) through their cysteine residue using sulfosuccinimidyl 4-(*p*-maleimidophenyl)butyrate as a coupling reagent (7). The peptide-BSA conjugates were coupled to Sepharose 4B according to the Pharmacia protocol. A synthetic peptide corresponding to amino acid sequence 735–752 from HIV-1 gp160 conjugated to BSA has been described elsewhere (8). Recombinant HIV IIIB gp160 expressed in vaccinia virus was a gift from J. R. Rusche (Repligen, Cambridge, MA) and natural HIV IIIB gp120 was provided by L. O. Arthur (National Cancer Institute, Bethesda, MD). Lysates of mature HIV IIIB virus were obtained from Organon Teknika–Cappel.

Production of Anti-gp160 Antibodies to Selected Epitopes in Different Animal Species. Six- to 8-week-old BALB/c mice and New Zealand White rabbits were injected with 100 and 500 μg, respectively, of peptide coupled to KLH emulsified in complete Freund's adjuvant (CFA). Mice were given a booster injection 3 weeks later with 100 μg of peptide–KLH

Abbreviations: HIV, human immunodeficiency virus; mAb, monoclonal antibody; Id, idiotype; ISCRI, interspecies cross-reactive Id; CRI, cross-reactive Id; Ab2β, anti-Id carrying the internal image of antigen; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin; RIPA, radioimmunoprecipitation assay; CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant.

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in incomplete Freund's adjuvant (IFA) and rabbits were given a booster injection 15 days later with 500 μg of peptide–KLH in IFA. Mice were bled 7 days after the booster injection and rabbits were bled 11 days after the last immunization. Adult baboons were immunized as follows: 100 μg of peptide coupled to KLH emulsified in stearyl tyrosine adjuvant (9) on days 1, 7, and 20; 1000 and 700 μg of peptide at days 69 and 178, respectively. The baboons were bled on days 34, 82, 96, 123, 151, 192, and 206. The immunization schedule for chimpanzees with recombinant HIV-1 gp160 has been described (10).

Characterization of Anti-Peptide Antibodies. Anti-peptide antibodies were purified over a column made of peptide–BSA conjugated to Sepharose 4B as described (11). The binding of affinity chromatography-purified anti-peptide antibodies to various antigens has been studied using a solid-phase sandwich and competition RIA as described (12, 13).

Radioimmunoprecipitation Assay (RIPA). The RIPA was performed as described (14) with some modifications. Briefly, mature virus lysates were labeled with ^{125}I precleared on Pansorbin cells, and 1–2 × 10^6 cpm were incubated overnight with 10 μg of antibodies. Complexes were brought down using Pansorbin cells. After heat denaturation, the proteins were separated on a 10% polyacrylamide gel.

Syncytium Formation Inhibition. This was done using 10^4 H9 cells productively infected with either the IIIB or the MN isolate and 10^5 uninfected HUT 78 cells as described (3).

Preparation of Anti-Id mAbs. BALB/c mice were immunized in the footpads with 100 μg of purified rabbit anti-gp160 antibodies specific for peptide 503–535 emulsified in CFA and given a booster injection 3 weeks later with the same amount emulsified in IFA. After a month, the mice were injected i.p. with 50 μg of rabbit antibodies per 24 hr for 3 days. Spleen cells were fused with SP2/0 nonsecreting myeloma cells. Hybrids were screened for binding to rabbit anti-peptide antibodies and to normal rabbit immunoglobulin as described (15). Three hybridomas of the $IgG_1\kappa$ class were cloned under stringent limiting dilution conditions, expanded *in vitro*, and mAbs were purified on a monoclonal rat anti-mouse κ light chain-Sepharose 4B column.

Id Expression. The binding of anti-Ids to anti-gp160 antibodies specific for peptide 503–535 from different animal species was performed as follows: microtiter plates were coated with 2 μg of anti-peptide antibodies, saturated with 2% BSA in phosphate-buffered saline, and then incubated with ^{125}I-labeled anti-Id antibodies. After 3 hr the plates were washed and radioactivity was assayed.

RESULTS

Inhibition of Syncytium Formation by Anti-Peptide Antiserum. The major goal of this study was to produce anti-Ids capable of mimicking HIV envelope epitopes. To achieve this goal, we prepared antibodies against various gp160 peptides and studied their ability to inhibit HIV-1 syncytium formation *in vitro* and to bind to native HIV-1 gp120 and gp160 proteins. Peptides corresponding to HIV-1 gp160-associated sequences 254–274, 420–445, 526–535, and 503–535 were coupled to carrier proteins and used for the immunization of two baboons, two rabbits, and five mice. In a pilot experiment, the anti-peptide sera were assayed for inhibition of syncytium formation caused by either HTLV IIIB or MN isolates. As depicted in Table 1, antibodies to peptide 503–535 produced in mice and rabbit but not their respective preimmune sera totally inhibited syncytium formation as did a human serum obtained from a HIV-infected patient. None of the three remaining anti-peptide antisera exhibited syncytium inhibitory activity *in vitro*.

Recognition of HIV Envelope Gene Products by Affinity-Purified Anti-Peptide Antibodies. For further analysis, anti-peptide antibodies were purified from the serum of mice, baboons, rabbits, and HIV-positive humans on peptide-BSA coupled to Sepharose 4B and assayed for binding to HIV-1 envelope proteins by RIPA. The data in Fig. 1 show that although the antibodies specific for peptides 254–274, 420–445, and 526–535 produced in rabbits and baboons did not precipitate HIV proteins (Fig. 1*A*), the antibodies specific for peptide 503–535 obtained from mice, rabbits, baboons, and humans precipitated a 120-kDa protein (Fig. 1*B*). Human antibodies precipitated three additional bands corresponding to p66, p55, and p41. This might be due to incomplete removal of undesired antibodies or to the presence of sticky immunoglobulin in the serum of HIV-positive individuals. The animal preimmune and normal human immunoglobulin did not precipitate any HIV proteins. The variability of band intensity observed among different species might reflect variations in the concentration and/or affinity of the antibody population recognizing the native envelope protein. These results clearly demonstrate that peptide immunization as well as HIV infection induced anti-503–535 antibodies that bound the native gp120 protein.

Table 1. Inhibition of syncytium formation by anti-peptides containing antiserum produced in different animal species

	Baboon		Rabbit		Mouse	
Inhibitor	IIIB	MN	IIIB	MN	IIIB	MN
Preimmune serum	–	–	–	–	–	–
Anti-254–274	–	–	–	–	–	–
Anti-420–445	–	–	–	–	–	–
Anti-526–535	–	–	–	–	–	–
Anti-503–535	–	–	+	+	+	+

Twenty microliters of a 1:10 dilution of serum was assayed for inhibition of syncytium formation between uninfected HUT 78 cells and H9 cells infected with either HIV-1 IIIB or MN isolates. A serum sample from a HIV-negative subject did not inhibit syncytium formation, whereas a sample from a HIV-positive subject inhibited syncytium formation mediated by either isolate. No cytotoxic effect of serum samples tested was observed on uninfected HUT 78 cells. For each peptide, antisera of two baboons, two rabbits, and a pool from five mice were tested. A + response indicates that >90% inhibition of syncytium formation was obtained with each individual serum tested.

Fine Specificity of the Purified Rabbit, Mouse, and Baboon Anti-gp160 Antibodies. In further experiments we studied the fine specificity of anti-peptide antibodies of various species by direct binding and competition RIA. As shown in Table 2, mice, rabbit, and baboon antibodies bind to 503–535-BSA, free peptide 503–535, 526–535-BSA, and gp160 but not to free peptide 526–535 or 735–752-BSA. The binding to 526–535-BSA but not to free peptide 526–535 may reflect the existence of antibodies produced against sulfosuccinimidyl 4-(*p*-maleimidophenyl)butyrate used for coupling peptides to BSA and KLH. The binding to peptide 503–535 was inhibited by free peptide 503–535 and 503–535-BSA. Furthermore, in the case of rabbit and mice antibodies, gp160 caused 55% and 52% inhibition, respectively, whereas in the case of baboons, only 25% inhibition was observed with 0.11 μM gp160 (not shown). Although the 526–535-BSA conjugate inhibited to some extent the binding of the anti-503–535 antibodies to 503–535-BSA, the free peptide did not. Again, this might be related to the common coupling reagent as stated above. Together these data indicate that the anti-503–535 antibodies are highly specific for the immunizing peptide and react with native gp160 protein.

Identification of the Antigen-Inhibitable ISCRI. Subsequent to immunization of mice with rabbit (R3) anti-503–535 antibodies, we obtained nine hybridomas that bound R3 anti-503–535 but not purified rabbit IgG obtained prior to immunization (data not shown). However, three mAbs, 3B10, 4C8, and 5A12 ($IgG_1\kappa$), bound purified anti-gp160 antibodies spe-

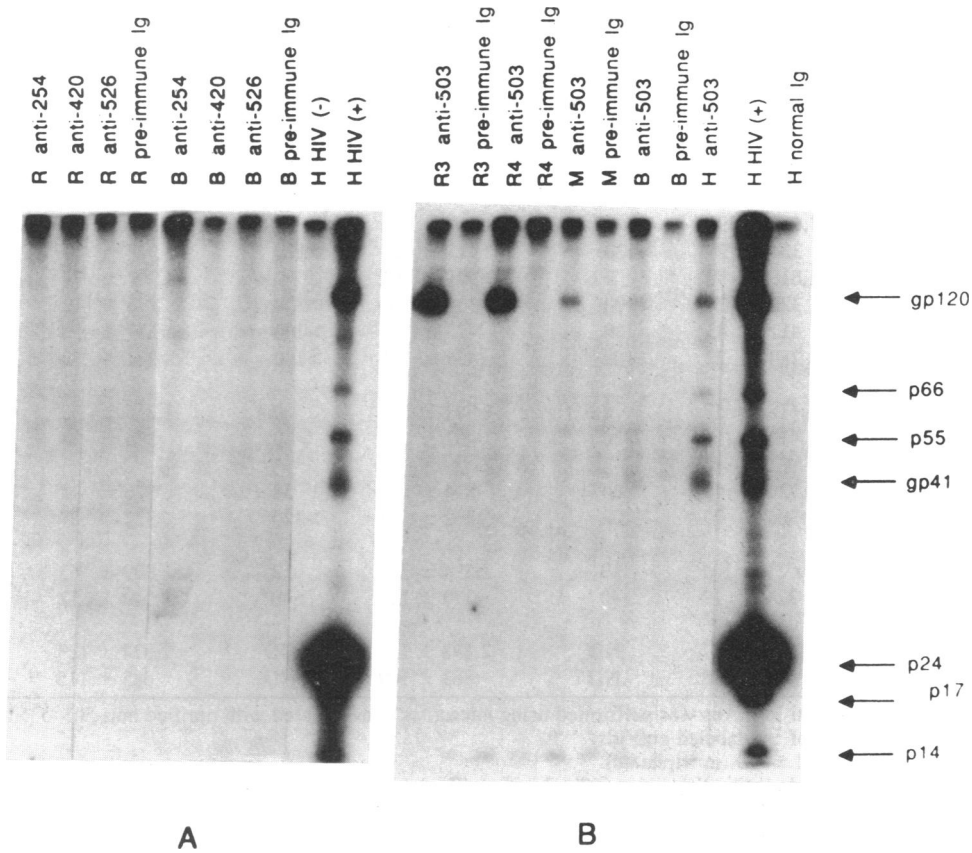

FIG. 1. Radioimmunoprecipitation of HIV-1 gp120 by affinity-purified anti-peptide antibodies. (*A*) Precipitation pattern with 10 μg of purified rabbit and baboon IgG obtained prior to immunization, with antibodies specific for peptides 254–274 (254), 420–445 (420), and 526–535 (526), or with 1 μl of human serum from a HIV-infected subject [H HIV (+)] and a normal healthy subject [H HIV (−)]. (*B*) Data obtained using 10 μg of purified anti-503–535 antibodies from rabbit 3 (R3 anti-503), rabbit 4 (R4 anti-503), mouse (M anti-503), baboon (B anti-503), and human (H anti-503). Ten micrograms of purified preimmune immunoglobulin from each animal and normal human immunoglobulin were included as negative controls. One microliter of human serum from a HIV-infected patient was used as a positive control.

cific for peptide 503–535 produced in two rabbits (R3 and R4), baboons, and mice and purified from the serum of HIV-infected patients along with chimpanzees immunized with gp160 (Table 3). The anti-Id mAbs did not bind preimmune or normal IgG of these animals nor did they bind to rabbit anti-735–752 or anti-526–535 antibodies. The direct binding was confirmed by a competition assay in which R3 anti-503–535 exhibited the highest inhibitory activity compared with baboon, mouse, and human anti-503–535 antibodies. These data clearly indicate that the anti-Ids specifically react with anti-503–535 antibodies of four different mammalian species. The data illustrated in Table 4 show that the Id–anti-Id interactions are inhibited by 503–535-BSA, free peptide 503–535, and gp160 but not by 526–535-BSA or BSA. The data also show that the binding of each anti-Id to anti-503–535 antibodies is inhibited by the other anti-Ids. However, the inhibition pattern of each anti-Id is distinct from the others, thereby indicating different clonal origin and specificity.

Table 2. Specificity of affinity-purified rabbit, mouse, and baboon anti-503–535 antibodies

Antigen or inhibitor	Rabbit		Mouse		Baboon	
	cpm*	μM†	cpm*	μM†	cpm*	μM†
BSA	114 ± 25	>1.10	175 ± 15	>1.10	107 ± 12	>1.10
735–752-BSA	81 ± 61	40.50	237 ± 13	>50.00	103 ± 14	>50.00
503–535-BSA	18,024 ± 229	2.80	8,812 ± 139	0.50	2,832 ± 75	1.50
503–535 free	15,546 ± 332	3.76	7,556 ± 169	0.70	2,528 ± 58	2.20
526–535-BSA	4,275 ± 181	>48.24	2,672 ± 323	70.00	1,640 ± 158	>100.00
526–535 free	81 ± 25	>48.24	448 ± 23	>11.00	157 ± 34	>100.00
gp160	2,552 ± 61	0.09	1,860 ± 110	0.10	380 ± 51	>0.11

The binding of anti-503–535 antibodies to various antigens was performed by incubating 10 ng of purified anti-503–535 antibodies on plates coated with antigen (2 μg/ml for free peptides 526–535 and 503–535, 5 μg/ml for 526–535-BSA, 503–535-BSA, and 735–752-BSA, and 10 μg/ml for gp160). Bound antibodies were revealed with ^{125}I-labeled, monoclonal rat anti-mouse κ light chain for mice, goat anti-rabbit IgG for rabbit, and goat anti-human immunoglobulin for baboon.

*Mean cpm bound ± SD.

†Concentration required for 50% inhibition. This assay was performed by simultaneously incubating ^{125}I-labeled anti-503–535 antibodies and various concentrations of competitor on 503–535-BSA-coated plates. The amount of ^{125}I-labeled anti-503–535 used in each case was preliminarily determined to give 80% binding to 503–535-BSA-coated plates. The molarity indicated for peptide-BSA conjugates is that of the coupled peptide and was calculated on the basis of a coupling ratio.

Table 3. Binding of murine anti-Id mAbs to chromatographically purified anti-gp120 antibodies specific for peptide 503–535 produced in various species

Origin of antibodies	Murine anti-Id mAb					
	3B10		4C8		5A12	
	cpm*	$\mu M^{\dagger}$	cpm*	$\mu M^{\dagger}$	cpm*	$\mu M^{\dagger}$
Rabbit						
R3 anti-503–535	27,618 ± 394	0.04*	17,339 ± 349	0.03	12,864 ± 185	0.03
R3 preimmune Ig	354 ± 24	>120	413 ± 26	>120	307 ± 11	>120
R4 anti-503–535	1,818 ± 58	2.6	3,901 ± 88	2.4	1,707 ± 77	3.5
R4 preimmune Ig	320 ± 26	>100	384 ± 16	>100	276 ± 12	>100
Anti-526–535	411 ± 43	>100	415 ± 77	>100	317 ± 56	>100
Anti-735–752	288 ± 22	>100	405 ± 63	>100	211 ± 19	>100
Murine						
Anti-503–535	1,412 ± 57	0.6	1,963 ± 156	0.4	1,215 ± 38	0.5
Preimmune Ig	412 ± 18	>30	452 ± 22	>30	313 ± 24	>30
Baboon						
Anti-503–535	726 ± 45	80	814 ± 26	58	926 ± 15	70
Preimmune Ig	306 ± 20	>120	391 ± 14	>120	322 ± 27	>120
Human‡						
Anti-503–535	1,023 ± 105	19	1,594 ± 213	12	909 ± 63	14
Normal Ig	324 ± 19	>40	279 ± 123	>40	240 ± 12	>40
Chimpanzee§						
Anti-gp160	2,836 ± 67	ND	2,898 ± 117	ND	2,824 ± 123	ND
Preimmune Ig	694 ± 78	ND	863 ± 109	ND	685 ± 115	ND

The binding of murine anti-Id mAbs was performed using microtiter plates coated with purified anti-503–535 antibodies (2 μg/ml) and 50,000 cpm of ^{125}I-labeled anti-Ids.

*cpm of anti-Id mAb bound ± SD (in triplicate).

†Concentration of anti-peptide antibodies giving 50% inhibition. The inhibition was assessed by solid-phase competition (16) in which 10,000 cpm of ^{125}I-labeled anti-Ids was simultaneously incubated with various concentrations of inhibitor on plates coated with purified rabbit (R3) anti-503–535 antibodies. ND, not done.

‡HIV-positive.

§Preimmune immunoglobulin and antiserum from a chimpanzee immunized with gp160 were incubated on microtiter plates coated with anti-Ids (2 μg/ml) and bound antibodies were revealed using ^{125}I-labeled goat anti-human immunoglobulin.

Induction of Anti-HIV Envelope Antibodies in Rabbits Immunized with Murine Anti-Id mAbs. Rabbits were immunized with 700 μg of purified anti-Id mAbs in CFA, were given a booster injection 3 weeks later with the same amount in IFA, and were bled weekly starting 11 days after the last immunization. All rabbits showed a high titer of anti-peptide antibodies. These antibodies were purified over a 503–535 BSA-Sepharose column and were assayed for binding to 503–535-BSA, gp120, gp160, and the three anti-Id mAbs. Table 5 clearly shows that the anti-Id-induced anti-503–535 antibodies bind to the peptide, gp120, and gp160 as do the antibodies obtained from a rabbit (R3) immunized with 503–535-KLH. They also bind to the anti-Ids used as immunogens. These results demonstrate the capacity of anti-Id mAb to elicit in rabbits anti-peptide antibodies that interact with the native envelope protein. We have suggestive preliminary indications that these antibodies exhibit neutralizing activity.

Table 4. Inhibition of Id–anti-Id interactions by peptide 503–535, gp160, and anti-Id mAbs

Inhibitor	Amount required for 50% inhibition,* μM or ng		
	3B10	4C8	5A12
Group 1			
503–535-BSA	2.50	13.50	2.50
526–535-BSA	>200.00	>200.00	>200.00
gp160	0.27	0.24	0.21
Group 2			
3B10	25	124	70
4C8	340	60	40
5A12	2400	110	70

The inhibition assay was performed as described in Table 3.

*The amount of inhibitor is μM in group 1 and ng in group 2.

DISCUSSION

It is well documented that anti-Ids bearing the internal image of antigen can induce antimicrobial immunity (6). In the present study, we report data demonstrating that antibodies to peptide 503–535 were able to bind the envelope protein and to inhibit syncytium formation mediated by two different isolates. Based on these observations and the fact that peptide 503–535 reacted with serum of HIV-infected patients (3), we used rabbit anti-503–535 antibodies to produce murine anti-Id mAbs. Three of nine mAbs recognize an antigen-inhibitable ISCRI shared by rabbit, mouse, baboon, and human anti-503–535 antibodies and chimpanzee anti-gp160 antibodies. The stronger binding of the anti-Ids to R3 anti-503–535 (the immunogen used to generate them) compared with their binding to anti-503–535 from different species indicates that interspecies cross-reactivity is based on sharing of a few critical amino acid residues that interact with the anti-Id antibodies.

The anti-Ids bind to antigen-specific antibodies from various species. In addition, the Id–anti-Id interactions are inhibited by nominal antigen. These two properties represent the best criteria to define anti-Ids carrying the internal image of an antigen (17). Indeed, injection of anti-Id mAbs into rabbits elicited antibodies that bind to peptide and to gp120 and gp160 proteins similar to those obtained from a rabbit immunized with 503–535-KLH. These results demonstrate that anti-Id mAbs raised against antibodies specific for gp120 epitopes are indeed able to elicit an anti-HIV humoral response (11, 18). On the other hand, these antibodies (induced by anti-Ids) bind more strongly to anti-Id mAbs than those obtained after immunization with 503–535-KLH and used to elicit the anti-Id. These data strongly suggest that all three anti-Id mAbs mimic peptide 503–535 and are of the Ab2β type. Further work is necessary to investigate the ability of

Table 5. Binding of antibodies obtained from rabbits immunized with peptide 503–535 or murine anti-Id mAbs

Binding to ligand	Immunogen*			
	503–535-KLH	5A12	4C8	3B10
BSA	34 ± 4	48 ± 3	71 ± 43	85 ± 26
735–752-BSA	111 ± 3	99 ± 1	127 ± 41	178 ± 24
503–535-BSA	12,254 ± 503	13,419 ± 73	12,422 ± 279	12,885 ± 357
gp120	1,318 ± 47	1,667 ± 43	1,323 ± 34	2,136 ± 24
gp160	5,731 ± 136	2,195 ± 182	2,708 ± 70	3,096 ± 59
5A12	2,553 ± 203	19,561 ± 636	17,923 ± 642	18,739 ± 287
4C8	7,672 ± 356	18,496 ± 471	17,489 ± 483	18,342 ± 516
3B10	3,921 ± 404	16,567 ± 527	14,753 ± 405	16,298 ± 358

Binding of 40 ng of chromatographically purified anti-503–535 antibodies obtained from rabbits immunized with 503–535-KLH or anti-Id mAbs. The plates were coated with BSA (5 μg/ml), 735–752-BSA (5 μg/ml), 503–535-BSA (5 μg/ml), gp120 (1 μg/ml), gp160 (10 μg/ml), and anti-Id mAbs 5A12, 4C8, and 3B10 (2 μg/ml).

*Bound antibodies were revealed with ^{125}I-labeled goat anti-rabbit IgG (100,000 cpm per well). Values are expressed as the mean ± SD of cpm bound (in triplicate).

these anti-Id antibodies to induce anti-HIV immunity in non-human primates.

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