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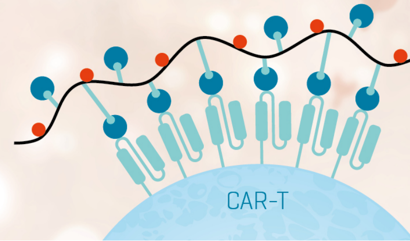

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ANTI-IDIOTYPIC ANTIBODY RESPONSE TO MONOCLONAL ANTI-CD4 PREPARATIONS IN NONHUMAN PRIMATE SPECIES[1]

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A series of mouse monoclonal anti-CD4 preparations was characterized for the ability to recognize overlapping epitopes on CD4 and to inhibit HIV/simian immunodeficiency virus (SIV) syncytium formation. Based on this characterization, mAb able to recognize CD4 epitopes overlapping the HIV binding site were selected and used to immunize nonhuman primates to elicit the production of specific anti-Id antibodies. Five baboons and five rhesus monkeys were immunized with either individual or a cocktail consisting of several monoclonal anti-CD4 preparations. All the nonhuman primates produced specific anti-Id that recognized either private or cross-reactive Id depending on the monoclonal anti-CD4 used to generate the anti-Id response. Inhibition assays were performed to ascertain the ability of: 1) soluble CD4 to inhibit the Id-anti-Id reaction and 2) the various anti-Id to inhibit the CD4-monoclonal anti-CD4 reaction. These studies demonstrated that some of the anti-Id recognized a cross-reactive Id that was associated with the Ag-combining site. In addition, some of the anti-Id weakly recognized SIV gp120 by Western blot analysis. These studies may be useful in designing experiments that may lead to a better understanding of the CD4-HIV gp120 interaction and to the production of Id and/or anti-Id reagents that might be used to manipulate this virus-receptor interaction.

Id are antigenic determinants expressed on the V region of the Ig molecule. Id represents the basis of the idiotypic network, first postulated independently by Lindenmann (1) and Jerne (2) and then experimentally demonstrated in several antigen-antibody systems (3). Through the production of anti-Id antibodies, the immune system is able to regulate the clonal dynamics, enhancing or suppressing the response to a given Ag. Anti-Id can be classified into two major categories, i.e., internal image or noninternal image anti-Id. Internal image anti-Id recognize Ag-combining site-related Id and are characterized by the ability to mimic the three-dimensional conformation of the original Ag. Noninternal image anti-Id recognize determinants that may or may be not associated with the Ag-combining site. Both classes of anti-Id have been used to induce in vivo the production of anti-anti-Id that are able to bind the nominal Ag without previous Ag exposure (4-6). In addition, anti-Id reagents have been used as probes to study the inheritance of Ig genes (7, 8), the structure of the antibody molecules (9), and the receptor-ligand interactions. Sege and Peterson (10) first demonstrated that anti-Id raised against antibodies to retinol-binding protein and insulin could mimic ligand epitopes and bind their respective receptors. Several studies have later confirmed the ability of anti-ligand antibodies, including anti-alprenolol (11), anti-thyrotropin (12), and anti-acetylcholine (13) antibodies, to elicit the production of anti-Id that bind their receptors and functionally mimic the biological action of the ligands (14). In addition, anti-Id able to mimic epitopes of viral antigens have been used to study and/or identify the cellular receptors of reovirus (15, 16), polyomavirus (17), and murine leukemogenic retroviruses (18). Conversely, in the EBV system, anti-Id able to bind virus epitopes have been generated against antibodies specific for the EBV receptor (19). These anti-Id have been used to identify the EBV binding site on the receptor. The present report describes the generation and characterization of anti-Id raised in nonhuman primates by immunization with mouse monoclonal anti-CD4.

The CD4 molecule is a glycoprotein expressed on the cell membrane of the helper/inducer T lymphocyte subset (20, 21) and of cells of the monocyte-macrophage lineage (22, 23), and eosinophils (24). CD4-bearing T cells provide activation signals to the cytotoxic/suppressor T lymphocyte (CD8) subset and participate in Ag recognition by interaction of the CD4 molecule with MHC class II molecules (25). In addition, the CD4 molecule is a cellular receptor for the HIV (26, 27) and SIV[3] (28). The physical interaction between CD4 and HIV or SIV occurs through the external virus envelope glycoprotein gp120. The interaction site has been mapped to a region within the outermost Ig V region-like domain of CD4 (29-31). The anti-Id generated in nonhuman primate species and characterized in the present study were produced against monoclonal anti-CD4 preparations recognizing CD4 epitopes overlapping the HIV/SIV gp120 binding site. The anti-Id recognized either a private or an Ag-combining site related cross-reactive Id. In addition, some of the

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[3] Abbreviations used in this paper: SIV, simian immunodeficiency virus; HBsAg, hepatitis B surface antigen; NGS, normal goat serum; T-PBS, Tween-20 containing PBS; sCD4, soluble CD4.

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anti-Id weakly recognized SIV gp120. This study provides information on the basis of the Id-anti-Id interactions that may be potentially useful in manipulating the CD4-HIV gp120 interactions.

MATERIALS AND METHODS

Inhibition of CD4-anti-Leu 3a binding. The ability of the various monoclonal anti-CD4 preparations to inhibit CD4-anti-Leu 3a binding was evaluated by using FITC-conjugated anti-Leu 3a (Becton Dickinson Monoclonal Center, San Jose, CA) and the CD4 expressing human T cell line, HPB-ALL. Briefly, 10^6 HPB-ALL cells were incubated with 5 μg of the various inhibitors and examined for the ability to inhibit a concentration of 10 ng of FITC-anti-Leu 3a from binding to the human $CD4^+$ cell line. CD4-anti-Leu 3a binding in the presence or absence of inhibitors was quantitated by using flow microfluorimetry.

Syncytium inhibition. H9 cells productively infected with either the HTLV-IIIB strain of HIV or the SIV_{mac}251 strain of SIV were used in this assay. A total of 100 μl of infected H9 cells (10^5/ml) was cocultured with 100 μl of HUT 78 (10^6/ml) in individual wells of a 96-well plate in the presence or absence of various monoclonal anti-CD4 reagents. A final concentration of 2 μg of each monoclonal anti-CD4 preparation was added to duplicate wells. Plates were incubated overnight at 37°C. Syncytium inhibition was evaluated by comparing the number of syncytium obtained in the presence of inhibitor to that obtained in the absence of inhibitor.

Preparation and purification of anti-Id antibodies. Five baboons and five rhesus monkeys were each immunized intramuscularly with 200 μg of mAb as an alum precipitate: 1) two baboons and three rhesus monkeys received anti-Leu 3a alone; 2) two baboons received L77 alone; 3) one baboon received a cocktail of monoclonal anti-CD4 preparations including anti-Leu 3a, L77, L34, L80; and 4) two rhesus monkeys received a cocktail including anti-Leu 3a, L34, and L110. The animals were immunized and bled bimonthly for a period of 6 mo. Serum obtained from each animal before the first injection served as a control. Nonhuman primate antisera obtained after the fifth injection were repeatedly adsorbed on a Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) column containing normal mouse γ-globulin until all detectable anti-isotypic and anti-allotypic reactivity was removed by methods previously described (32). Baboon IgG was purified from the anti-Id fraction by using a *Staphylococcus* protein A agarose column.

Direct binding assays. The presence of anti-isotype and anti-allotype, along with anti-Id antibodies, was determined by a direct binding ELISA using mAb preparations with specificities for CD4 and HBsAg. A total of 200 ng of various mAb was diluted in 50 μl of borate buffered saline, added to each well 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 nonspecific binding sites with 10% NGS for 30 min at 37°C, the plate was washed with 0.01% T-PBS. Serial dilutions in 10% NGS of the various anti-Id preparations were added to duplicate wells for 1 h at 37°C, and the plate was washed with T-PBS. To detect specific binding of the anti-Id produced in the non-human primate species, biotinylated goat anti-human γ-globulin (Vector Laboratories, Burlingame, CA) was diluted in 10% NGS and incubated in each well for 1 h at 37°C. After washing with T-PBS, streptavidin conjugated to horseradish peroxidase (Bethesda Research Labs, Gaithersburg, MD) was added for 25 min at 25°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) (Sigma Chemical Co., St. Louis, MO) in 0.1 M citrate adjusted to pH 4.0 with 1 N NaOH. Immediately before use, 0.01% H_2O_2 v/v was added. The addition of 5% SDS was used to stop the reaction. OD of each well was determined on an automatic ELISA plate reader at 410 nm (MR600; Dynatech Laboratories, Inc., Alexandria, VA). Background levels were subtracted from each well of the microtiter plate.

To ascertain whether the anti-Id preparations recognized cross-reactive structures shared by anti-Leu 3a and the CD4 molecule and therefore would bind both anti-Leu 3a and the CD4 molecule, a direct binding ELISA was performed by coating microtiter wells with 100 ng of recombinant sCD4 (kindly provided by Dr. G. Cianciolo, Genetech, Inc., South San Francisco, CA) in 50 μl of BBS overnight at 4°C. After blocking of nonspecific binding sites with 10% NGS, the ELISA plate was washed with T-PBS and a 1/20 dilution of the various anti-Id preparations was added to duplicate wells. The remainder of the assay is as described above for the other direct binding assays.

Detection of private and cross-reactive Id. To determine the ability of the various anti-Id to recognize either private or cross-reactive Id, a direct binding assay was performed as described above. Microtiter wells were coated with 200 ng of the various monoclonal anti-CD4 preparations, and serial dilutions of the anti-Id were added. Endpoint titers were considered to be the reciprocal of the highest anti-Id dilution that resulted in an OD value that was more than the value obtained with the negative control mouse mAb specific for HBsAg (designated A2.1). The detection of a cross-reactive anti-CD4 Id by the various anti-Id preparations was based on an endpoint titer of more than 200 (reciprocal dilution). A monoclonal anti-CD4 preparation that exhibited an endpoint titer of less than 100 with a given anti-Id was considered not to express a cross-reactive anti-CD4 Id.

Competitive inhibition assays. To determine whether the Id-anti-Id binding reaction was Ag inhibitable, a competitive inhibition assay was performed. A total of 200 ng of the mAb preparations in 50 μl of BBS was coated onto individual wells of the ELISA plate overnight at 4°C. Nonspecific binding sites were blocked by adding 10% NGS for 30 min at 37°C and the plate was washed in T-PBS. A dilution in 10% NGS of anti-Id preparations corresponding to the linear part of the Id-anti-Id binding curve was mixed with different concentrations of soluble CD4 or an irrelevant inhibitor (BSA), was added to triplicate wells in 50 μl amounts and allowed to incubate overnight at 4°C. After washing with T-PBS, biotinylated goat anti-human γ-globulin was added to each well for 1 h at 37°C. The addition of steptavidin conjugated to horseradish peroxidase and substrate was the same as described for the direct binding ELISA.

To evaluate the ability of the anti-Id preparations to inhibit the binding of anti-Leu 3a to soluble CD4, a second competitive inhibition assay was used. A total of 200 ng of CD4 or a control Ag, HBsAg, was adsorbed on the solid phase overnight at 4°C. After blocking nonspecific binding sites with 10% NGS, the ELISA plate was washed with T-PBS. A constant amount of anti-Leu 3a or a mouse mAb preparation specific for HBsAg that corresponded to the linear portion of the Ag-Id binding curve was mixed with serial dilutions of the anti-Id preparations and added to triplicate microtiter wells. The plate was incubated overnight at 4°C. After washing with T-PBS, HRP-goat anti-mouse gamma-globulin (gamgg) (Kirkegaard and Perry labs, Inc., Gaithersburg, MD) was added to each well for 1 h at 37°C. The addition of substrate was performed as described in other assays. For all the competitive inhibition assays, the percent of inhibition was calculated with the formula:

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

Western blot analysis. Cell culture supernatants were harvested from SIV_{mac}251 infected HUT 78 cells in peak log phase. The fluid was filtered through a 0.45-μm filter and the virus pelleted through a 20% sucrose phase in a Beckman SW28 rotor (Beckman Instrument Co., Fullerton, CA) for 2 h at 4°C. The virus pellet was resuspended in 1 ml of virus lysis buffer (0.05 M Tris-HCl, pH 7.4, 1% Triton X-100, and 0.1% SDS) and incubated overnight at 4°C with 1 ml (10% w/v) of lentil lectin Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ). The bound fraction was washed four times and resuspended in sample buffer (0.08 M Tris-HCl pH 6.8, 2% SDS, 10% glycerol, and bromophenol blue) and the sample boiled for 3 min before the proteins were separated on a 12% SDS-PAGE system as described by Laemmli (33). The proteins were passively transferred to nitrocellulose membranes, nonspecific binding was blocked by incubation with 3% BSA (fraction V, Sigma) at 37°C for 1 h and used directly or frozen at −20°C. Strips were reacted with 150 μl of a 1/10 dilution of anti-Id containing sera in 2 ml of 0.2% Tween 20 in PBS overnight at 4°C, washed extensively and developed with biotinylated sheep anti-human antibody and then streptavidin according to the manufacturer's specification (Amersham Corp., Arlington Heights, IL). Diaminobenzedine (Sigma) was used as the substrate. These methods have been previously described (34).

RESULTS

Monoclonal anti-CD4 preparations recognize CD4 epitopes that overlap HIV/SIV gp120 binding site. A panel of mouse monoclonal anti-CD4 preparations was characterized for the ability to recognize overlapping epitopes on the CD4 molecule and inhibit HIV and SIV syncytium formation. The characteristics of the various monoclonal anti-CD4 preparations are summarized in Table I. Anti-Leu 3a was able to inhibit both HIV and SIV syncytium formation. In addition, it has been previously demonstrated that anti-Leu 3a inhibits the CD4-HIV gp120 binding (35). These data suggest that anti-Leu 3a recog-

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TABLE I
Characteristics of mouse monoclonal anti-CD4 preparations

Antibody Designation	Isotype	Inhibition of CD4-Anti-Leu 3a Binding	Inhibition of HIV Syncytium Formation	Inhibition of SIV Syncytium Formation
Anti-Leu 3a	IgG1	+[a]	+[b]	+[b]
L34	IgG1	+	+	+
L69	IgG1	+	−	−
L77	IgG1	+	−	−
L80	IgG1	+	+	+
L83	IgG1	−	+	+
L88	IgG2a	+	+	±
L92	IgG1	−	−	−
L93	IgG1	+	+	+
L104	IgG2a	−	+	+
L110	IgG1	±	+	+
L112	IgG2b	−	−	−
L198	IgG1	+	+	+
L199	IgG2a	+	+	±
L200	IgG1	+	+	+
L202	IgG1	+	+	+
L204	IgG2a	+	−	−
L206	IgG1	+	±	±
L208	IgG1	+	+	−

[a] + indicates more than 90% inhibition of binding; ± indicates weaker levels of inhibition (between 40 and 60%); − indicates lack of inhibition (less than 10%). All inhibitors were examined at a final concentration of 5 μg.
[b] + indicates more than 90% inhibition, ± indicates weaker level of inhibition (between 50 and 80%). − indicates lack of inhibition of syncytium formation. Each inhibitor was examined at a final concentration of 2μg.

nizes an epitope on the CD4 molecule overlapping the HIV/SIV binding site. The majority of the monoclonal anti-CD4 preparations inhibited the CD4-anti-Leu 3a binding, indicating that they recognized CD4 epitopes that overlap the epitope recognized by anti-Leu 3a. In addition, certain monoclonal anti-CD4 exhibited the ability to inhibit both HIV and SIV syncytia formation. Together, these data indicate that some of the monoclonal anti-CD4 preparations recognized overlapping epitopes that are involved in HIV/SIV gp120 binding to the CD4 molecule.

Nonhuman primate antibodies raised against monoclonal anti-CD4 preparations recognize Id determinants. Five monoclonal anti-CD4 preparations recognizing CD4 epitopes overlapping the HIV/SIV gp120 binding site to various degrees were selected and used to immunize nonhuman primates to elicit the production of specific anti-Id. Five baboons and five rhesus monkeys received six monthly intramuscular injections of monoclonal anti-CD4 preparations as an alum precipitate. The monoclonal anti-CD4 were administered either individually or as a cocktail. Serum obtained from each animal after the fifth injection was exhaustively adsorbed over a Sepharose 4B column containing a pool of normal mouse Ig to remove all detectable anti-isotypic and anti-allotypic activity. The resulting anti-Id preparations were tested for specificity using a direct binding ELISA. The immunizing monoclonal anti-CD4, along with an irrelevant mouse mAb, were adsorbed on the solid phase and serial dilutions of the various anti-Id were added. All the anti-Id preparations bound their respective monoclonal anti-CD4, whereas no significant reactivity was observed with A2.1, a mouse mAb specific for HBsAg that expressed an isotype similar to that of the immunizing monoclonal anti-CD4. Serum obtained from each animal before immunization failed to bind the monoclonal anti-CD4 Id. Representative binding curves of anti-Id generated in baboons binding their respective Id are shown in Figure 1. At a reciprocal dilution of 50, all the anti-Id bound their respective anti-CD4 Id with an OD_{410} value of more than 0.80, whereas at a similar dilution an OD value of less than 0.10 was obtained using the control mAb, A2.1. Figure 2 shows representative binding curves of anti-Id generated in rhesus monkeys binding their respective immunizing Id. At a reciprocal dilution of 50, the anti-Id bound their respective anti-CD4 Id with an OD_{410} value equal to or more than 1.0, whereas at a similar dilution an OD value of less than 0.10 was obtained using the control mAb, A2.1. These data indicate that the nonhuman primate anti-Id preparations specifically recognized Id determinants expressed on the monoclonal anti-CD4 preparations used in their generation.

Nonhuman primate anti-Id recognize either private or cross-reactive Id. Numerous studies have demonstrated that Id can be unique to a given antibody or can be shared by antibodies of a given specificity (36, 37). The first category of Id is referred to as private Id (IdI). Shared Id are also denoted cross-reactive Id (IdX) (38). To ascertain whether the nonhuman primate anti-Id recognized IdI or IdX, a direct binding ELISA was performed. Monoclonal anti-CD4 preparations were adsorbed to the solid phase and serial dilutions of the anti-Id were added. The anti-Id reactivity to the various monoclonal anti-CD4 preparations is shown in Table II. Anti-Id induced by immunization with L77 (X1616 and X3807) recognized only this anti-CD4 preparation, whereas anti-Id induced by immunization with either anti-Leu 3a or the cocktail anti-CD4 preparations recognized, to various degrees, a panel of different monoclonal anti-CD4 preparations. These results indicate that anti-Id from nonhuman primates immunized with L77 recognized primarily an IdI, whereas anti-Id from animals immunized with either anti-Leu 3a or the cocktail recognize an IdX.

Cross-reactive Id is Ag-combining site related. To determine whether the nonhuman primate anti-Id recognized Id determinants associated with the Ag-combining site, competitive inhibition assays were performed. In a first set of experiments, anti-Leu 3a or L77 were bound to the solid phase. Different concentrations of recombinant sCD4 were mixed with a constant dilution of anti-Id and added to the microtiter wells. The resulting inhibition curves are shown in Figure 3. A total of 250

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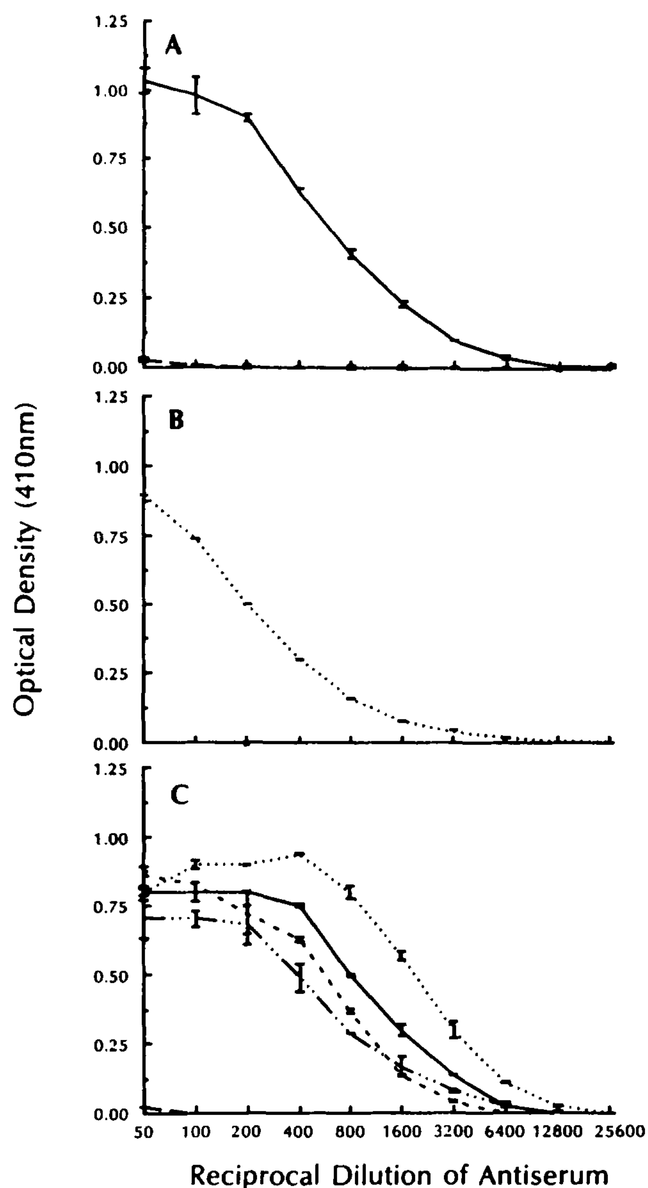

Figure 1. Representative binding curves of anti-Id responses in baboons to the monoclonal anti-CD4 preparations. *A*, Baboon X3424 (anti-Leu 3a); *B*, baboon X3807 (L77); *C*, baboon 2004 (cocktail including anti-Leu 3a, L77, L34, L80); binding to anti-Leu3a (——), L77 (···), L34 (- - -), L80 (- ··· -), A2.1 (—— ——). Each *point* represents the mean of duplicate values. The *vertical bars* represent the range of values.

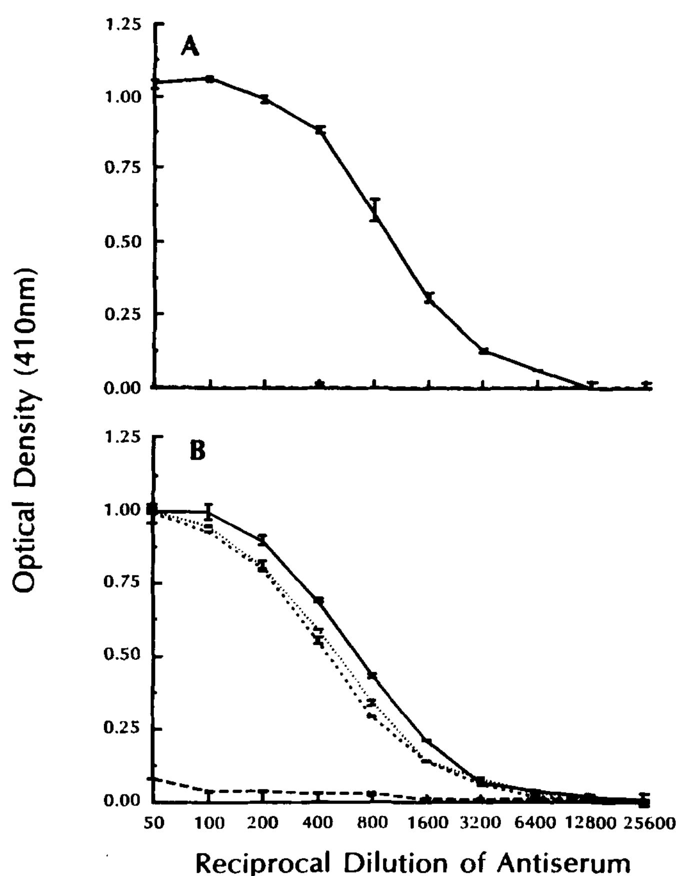

Figure 2. Representative binding curves of anti-Id responses in rhesus monkeys to the monoclonal anti-CD4 preparations used. *A*, Rhesus monkeys 7913 (anti-Leu 3a); *B*, rhesus monkey 7910 (cocktail including anti-Leu 3a, L34, L110) binding to anti-Leu3a (——), L34 (···), L110 (- - -), A2.1 (—— ——).

ng of sCD4 was able to inhibit the anti-Leu 3a-anti-Id reaction by more than 70% (range 74 to 95% for the eight anti-Leu 3a-anti-Id reactions), whereas no significant inhibition was observed by using a similar amount of an irrelevant inhibitor. The two L77-anti-Id reactions were not significantly inhibited by using a similar concentration of sCD4 (23 and 9%). These data indicated that the IdX is Ag-combining site related, whereas the private Id does not appear to be associated with the Ag-combining site.

Inasmuch as the anti-Id preparations generated in these nonhuman primate species are polyclonal, the possibility exists that the anti-Id preparations recognized an Id determinant within the anti-Leu 3a Ag-combining site that was distinct from the IdX detected on the other monoclonal anti-CD4 preparations. To confirm the association of the IdX with the Ag-combining site, a single anti-Id preparation from a baboon immunized with anti-Leu 3a (X3424) was selected for additional inhibition assays. Microtiter wells were coated with the monoclonal anti-CD4 preparations expressing the IdX (see data from Table II), and sCD4 was used to inhibit the binding of the anti-Id to the various monoclonal anti-CD4. As shown in Table III, sCD4 inhibited the binding of the anti-Id to the various monoclonal anti-CD4 preparations. Less than 500 ng of soluble CD4 were able to inhibit the anti-Id-monoclonal anti-CD4 Id reactions by 50%. No inhibition was observed when a similar amount of an irrelevant inhibitor (BSA) was used. These data confirmed the association of the IdX with the Ag-combining site. However, the possibility exists that inhibition of the Id-anti-Id reaction by the Ag may result from allosteric effects induced by the CD4 molecule binding to the anti-CD4 preparations. This inhibition could also have resulted from steric hindrance of the Id-anti-Id reaction by soluble CD4. Thus, we attempted a more rigorous determination of the Id localization by performing assays to determine if the anti-Id is able to inhibit the CD4-monoclonal anti-CD4 reaction.

In the next set of experiments, inhibition of the CD4-anti-Leu 3a binding was attempted by using the various anti-Id. Soluble CD4 was adsorbed on the solid phase, and a constant amount of anti-Leu 3a mixed with serial dilutions of the anti-Id was added to the CD4 coated microtiter wells. A 1/10 dilution of the anti-Id recognizing the IdX was able to inhibit the CD4-anti-Leu 3a binding by more than 90% (Fig. 4). A similar dilution of preim-

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TABLE II
Detection of cross-reactive and private anti-CD4 Id by anti-Id generated in nonhuman primate species

Antibody Designation	Baboon Anti-Id				
	X1616 L77[a]	X3807 L77	X2004 anti-Leu 3a L77, L34, L80	X4289 anti-Leu 3a	X3434 anti-Leu 3a
Anti-Leu 3a	<100	<100	3200[b]	6400	3200
L34	<100	<100	1600	800	800
L69	<100	<100	<100	<100	<100
L77	3200	3200	6400	<100	<100
L80	<100	<100	3200	400	1600
L83	<100	<100	<100	<100	<100
L88	<100	<100	<100	<100	<100
L92	<100	<100	<100	<100	<100
L93	<100	<100	3200	1600	1600
L104	<100	<100	<100	<100	<100
L110	<100	<100	1600	400	400
L112	<100	<100	<100	<100	<100
L198	<100	<100	1600	1600	1600
L199	<100	<100	3200	1600	1600
L200	<100	<100	1600	400	800
L202	<100	<100	3200	3200	3200
L204	<100	<100	<100	<100	<100
L206	<100	<100	1600	1600	1600
L208	<100	<100	1600	1600	1600
	Rhesus Monkey Anti-Id				
	7910 Anti-Leu 3a L34, L110	7911 Anti-Leu 3a L34, L110	7913 Anti-Leu 3a	7914 Anti-Leu 3a	7915 Anti-Leu 3a
Anti-Leu 3a	3200	1600	3200	3200	3200
L34	3200	1600	800	800	400
L69	<100	<100	<100	<100	<100
L77	<100	<100	<100	<100	<100
L80	3200	1600	1600	400	800
L83	<100	<100	<100	<100	<100
L88	<100	<100	<100	<100	<100
L92	<100	<100	<100	<100	<100
L93	3200	1600	1600	1600	1600
L104	<100	<100	<100	<100	<100
L110	3200	1600	800	400	800
L112	<100	<100	<100	<100	<100
L198	1600	1600	800	400	800
L199	1600	1600	800	800	400
L200	1600	1600	400	200	400
L202	3200	3200	1600	800	1600
L204	<100	<100	<100	<100	<100
L206	1600	800	800	400	400
L208	1600	1600	800	400	800

[a] Immunizing mAb.
[b] Represents reciprocal endpoint titers.

mune sera obtained from the nonhuman primates before immunization failed to inhibit the same reaction. Anti-Id preparations from animals immunized with L77 were used as an additional control. These two anti-Id failed to significantly inhibit the CD4-anti-Leu 3a reaction. In addition, the anti-Id recognizing the IdX failed to inhibit the binding of A2.1 to HBsAg, further indicating that inhibition of the CD4-anti-Leu 3a binding was not due to the presence of a residual anti-isotypic and/or anti-allotypic activity.

To exclude the possibility that inhibition of either the Id-anti-Id or the CD4-anti-CD4 reaction was due to the recognition by the anti-Id of cross-reactive structures or conformation shared by CD4 and anti-Leu 3a, a direct binding assay was performed. This possibility existed because CD4 is a member of the Ig supergene family and appears to exhibit Ig fold tertiary structure similar to the monoclonal anti-CD4. Soluble CD4 was adsorbed to the solid phase, and the various nonhuman primate anti-Id preparations were added. No binding of the anti-Id to sCD4 was observed (data not shown). These data provide additional evidence for the conclusion that the IdX is Ag-combining site related.

Nonhuman primate anti-Id recognize SIV gp120. The ability of the anti-Id to recognize an IdX associated with the Ag-combining site suggested that an antibody population exhibiting internal image activity may have been produced during the anti-Id response. Inasmuch as the monoclonal anti-CD4 preparations used to immunize the nonhuman primates recognize CD4 epitopes overlapping the HIV/SIV binding site, internal image anti-Id should mimic the three-dimensional structure of these CD4 epitope(s) and possibly bind HIV/SIV gp120. Western blot analysis was performed to determine the ability of the anti-Id to bind SIV gp120. Lentil-lectin purified glycoproteins from SIV were separated by SDS-PAGE and passively transferred to nitrocellulose. Strips were incubated with sera obtained from sequential bleeds of the nonhuman primates immunized with the monoclonal anti-CD4 preparations. Anti-Id-containing sera obtained 14 days after the fifth immunization from the baboon immunized with the cocktail of monoclonal anti-CD4 and 28 days after the fifth immunization from the three rhesus monkeys immunized with anti-Leu 3a alone showed weak reactivity with SIV gp120. Sera obtained from previous bleeds were unable to bind SIV gp120. Sera from baboons

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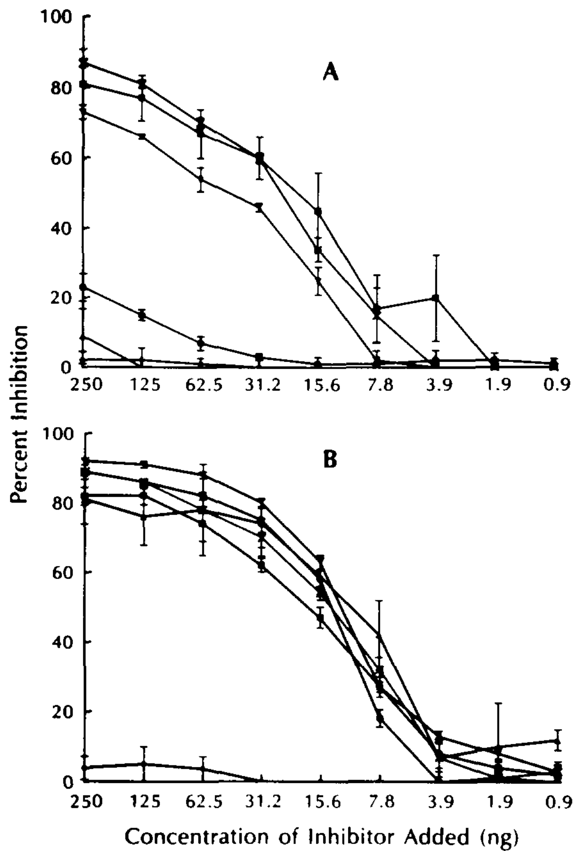

Figure 3. Inhibition curves of the various anti-Id binding to anti-Leu 3a or L77 by using soluble CD4. *A*, Anti-Id generated in baboons: X1616 (●), X3807 (▲), X3424 (■), X4289 (X), X2004 (▼); *B*, anti-Id generated in rhesus monkeys: 7910 (●), 7911 (▲), 7913 (■), 7914 (X), 7915 (▼). Each *point* represents the mean of triplicate determinations. The *vertical bars* represent the range of values. The means of the values obtained by using an irrelevant inhibitor to inhibit the various Id-anti-Id reactions are shown (♦). The monoclonal anti-CD4 preparations used to generate the various nonhuman primate anti-Id are indicated in Table II.

TABLE III

Inhibition of X3424 anti-Id binding to monoclonal anti-CD4 antibodies by using soluble CD4

Antibody Designation	I_{50} (ng)
Anti-Leu 3a	25[a]
L34	40
L80	414
L93	81
L110	35
L198	30
L199	85
L200	107
L202	44
L206	37
L208	24

[a] Represents the concentration of soluble recombinant CD4 that inhibits the binding of the anti-Id to the various monoclonal anti-CD4 by 50%.

immunized with anti-Leu 3a or L77 alone, and from rhesus monkeys immunized with the cocktail of monoclonal anti-CD4 preparations failed to bind SIV gp120. Figure 5 shows a representative Western blot analysis performed by using serum from the baboon immunized with the cocktail of monoclonal anti-CD4 antibodies. It is apparent that the anti-Id containing serum specifically recognized SIV gp120. The specificity of the SIV gp120 binding is demonstrated by the inability of preimmune serum obtained from the same animal to bind SIV glycoproteins. However, anti-Id containing sera obtained after the sixth immunization failed to exhibit SIV gp120 reactivity. These data indicated that some of the nonhuman primates were able to transiently produce anti-Id exhibiting SIV gp120 reactivity and, potentially, internal image anti-Id activity based on serological mimicry of CD4 epitopes involved in binding gp120.

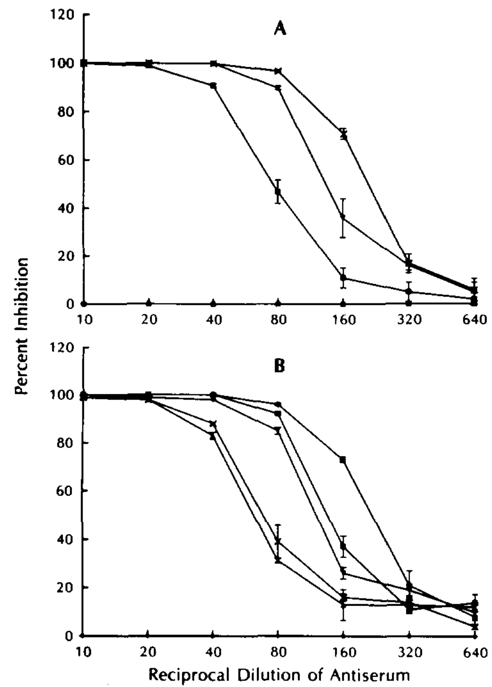

Figure 4. Inhibition curves of anti-Leu 3a binding to soluble CD4 by using anti-Id generated in nonhuman primates. *A*, Anti-Id generated in baboons: X1616 (●), X3807 (▲), X3424 (■), X4289 (X), X2004 (▼); *B*, anti-Id generated in rhesus monkeys: 7910 (●), 7911 (▲), 7913 (■), 7914 (X), 7915 (▼). The means of the values obtained by using the anti-Id generated in nonhuman primates to inhibit the binding of a monoclonal antibody exhibiting HBsAg specificity to HBsAg are indicated (♦).

DISCUSSION

Mouse monoclonal anti-CD4 preparations were characterized for the ability to recognize overlapping epitopes on CD4 and to inhibit HIV/SIV syncytium formation. Five monoclonal anti-CD4 preparations exhibiting the ability to recognize CD4 epitopes involved in HIV/SIV gp120 binding were selected and used, either alone or as a cocktail, to immunize nonhuman primates to elicit an anti-Id response. All the animals produced specific anti-Id able to recognize the respective immunizing anti-CD4 Id. The monoclonal anti-CD4 were administered intramuscularly as an alum precipitate, which is the only adjuvant currently approved for use in humans. Consequently, our results suggest that only low anti-Id titers were elicited. A stronger anti-Id response could have been produced by immunization with more potent adjuvants, such as CFA, and/or by coupling the monoclonal anti-CD4 to carrier proteins, such as keyhole limpet hemocyanin (39). However, because this study was initially designed to assess the feasibility of monoclonal anti-CD4 preparations as Id-based vaccines for HIV in nonhuman primate species, relevance and possible extrapolation of the anti-Id response to humans was desired, rather than maximum anti-Id response.

Anti-Id from nonhuman primates immunized with L77 recognized an IdI associated only with L77, whereas anti-Id from animals immunized with either anti-Leu 3a or the cocktail recognized an IdX associated with anti-Leu

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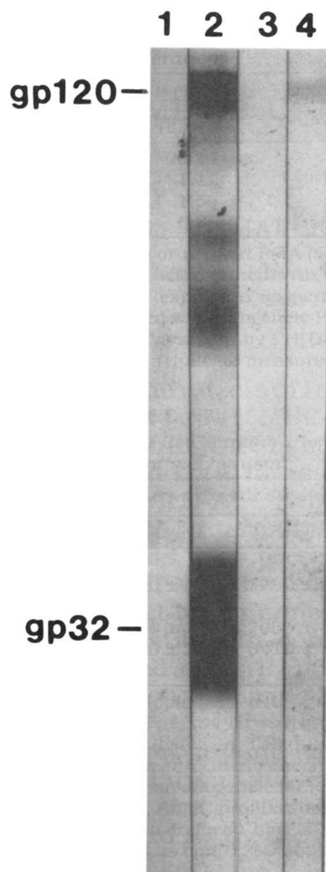

Figure 5. Western blot analysis showing the binding of the anti-Id generated in baboon X2004 to SIV gp120. SIV glycoproteins were purified by incubation of the virus lysate with lentil lectin after denaturation and separation by SDS-PAGE. SIV glycoproteins were passively transferred to nitrocellulose paper. Strips were reacted with serum from an SIV-seronegative rhesus monkey representing the negative control (*lane 1*), serum from an SIV_{mac}-infected rhesus monkey representing the positive control (*lane 2*), serum from baboon X2004 obtained before immunization with the cocktail of monoclonal anti-CD4 preparations (*lane 3*), serum from baboon X2004 obtained after the fifth injection with the cocktail of monoclonal anti-CD4 preparations (*lane 4*).

3a and a panel of different monoclonal anti-CD4 preparations. Competitive inhibition assays were performed to ascertain whether the IdI and the IdX were Ag-combining site related or not. Soluble CD4 was able to inhibit the anti-Leu 3a-anti-Id reaction. In addition, sCD4 inhibited the binding of an anti-Id preparation, generated by immunizing with anti-Leu 3a, to all the monoclonal anti-CD4 preparations expressing the IdX. No significant inhibition of the L77-anti-Id reaction was observed by using sCD4. These results indicated that the IdX and the IdI are Ag-combining-site and non-Ag-combining-site related, respectively. To confirm the association of the IdX with the Ag-combining site, the ability of the nonhuman primate anti-Id preparations to inhibit the CD4-anti-Leu 3a binding was assessed by performing competitive inhibition assays. All the anti-Id preparations generated by immunizing with either anti-Leu 3a or the cocktail of monoclonal anti-CD4 were able to inhibit the Ag-Id reaction. Anti-Id generated by immunization with L77 failed to inhibit the CD4-anti-Leu 3a reaction. The presence of reciprocal inhibition (inhibition of the Id-anti-Id reaction by sCD4 and inhibition of the CD4-anti-Leu 3a reaction by anti-Id) strongly suggested that the cross-reactive Id is Ag-combining site related. However, the CD4 molecule is a member of the Ig gene superfamily and exhibits sequence homology with the antibody molecule (40). In particular, the gp120 binding site has been mapped to a region within the outermost V-like domain of CD4 (30, 31). This region presents structural similarities with the second CDR of the κ-chain of the Ig molecules. It could be possible that inhibition of the CD4-anti-Leu 3a reaction and the Id-anti-Id reaction was due to the anti-Id recognition of tertiary Ig structures shared by anti-Leu 3a and CD4. To assess this possibility, the primary amino acid sequences of anti-Leu 3a V region and CD4 were compared and direct binding assays were performed to evaluate the ability of the anti-Id to recognize sCD4. No primary amino acid sequence homology was found between anti-Leu 3a and CD4 (R. Attanasio and R. C. Kennedy unpublished results) and no binding of the anti-Id to sCD4 was observed.

The ability of the anti-Id to recognize an IdX associated with the Ag-combining site might be an indication of the presence of an antibody population exhibiting internal image anti-Id activity. Inasmuch as the anti-Id were generated by immunizing with mAb binding CD4 epitopes overlapping the gp120 binding site, internal image anti-Id should mimic these epitopes and bind gp120. Western blot analysis showed that some of the anti-Id preparations weakly and transiently recognized SIV gp120. These results are not surprising. During a polyclonal anti-Id response, a small fraction of the total antibodies may exhibit internal image activity. In the tobacco mosaic virus system, Urbain et al. (41) have estimated that only 15% of the anti-Id are internal image antibodies. Inasmuch as the anti-Id response in nonhuman primate species was present at a low titer, it may be possible that even small fluctuations in the anti-Id response could influence the anti-gp120 reactivity. In addition, recent studies using mutational analysis suggest that the CD4 epitope recognized by anti-Leu 3a does not precisely overlap with the gp120 binding site (42, 43). It may be possible that the weak anti-gp120 response is the result of an only partial mimicry of the CD4 site involved in gp120 binding. Some of the monoclonal anti-CD4 used to immunize the nonhuman primates were able to bind the CD4 molecule expressed on the surface of lymphocytes from baboons and rhesus monkeys (J. S. Allan unpublished results). The CD4 site involved in gp120 binding appears therefore highly conserved in baboons, rhesus monkeys, and humans. The anti-Id produced by immunization with the monoclonal anti-CD4 preparations might in theory elicit the endogenous production of anti-anti-Id with specificity for CD4. Thus, it may be possible that specific mechanisms become operational to down-regulate the production of anti-Id exhibiting internal image activity to inhibit the production of anti-anti-Id with binding specificity for a self Ag, in this case CD4. However, it might be possible that the SIV gp120 reactivity is not the result of internal image activity.

According to Gaulton and Greene (44), the ability of noninternal image anti-Id to recognize receptor epitopes might be explained by the existence of cross-reactive structures shared by the Id-bearing antibody and the physiologic receptor of the ligand. The receptor epitope recognized by the noninternal image anti-Id may be distinct from both the epitope recognized by the paratope of the Id-bearing antibody and the receptor site involved in ligand binding. In our experimental system, cross-reac-

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tive structures might be shared by SIV gp120 and anti-Leu 3a. Comparison of the primary amino acid sequences of $SIV_{mac}251$ gp120 and both H and L chains of anti-Leu 3a showed no significant homology. However, cross-reactive structures might exist at the three-dimensional level.

Based on these results, defining the mechanism(s) leading to the anti-Id recognition of gp120 is difficult. The presence of antibodies exhibiting internal image activity within the polyclonal anti-Id response in nonhuman primate species may be possible. Further studies are required to determine the means by which anti-Id recognize gp120 and to assess the potential application of these results for manipulating the CD4-HIV gp120 interaction.

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