Production of Multimeric Forms of CD4 through a Sugar-based Cross-linking Strategy*

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We have developed a three-step cross-linking procedure that is specifically targeted at the carbohydrate on a protein and applied it to CD4 as a model system for studying the role of multivalent interactions in function. In the first step CD4 was oxidized with potassium iodate, creating aldehydes that served as targets for the subsequent chemistry. Next the aldehydes were modified with cystamine, converting the reactive group into a thiol. Finally cross-linking through the thiol moiety was generated with the homobifunctional cross-linker bismaleimidohexane. With this procedure, approximately 60% of the CD4 was converted into higher molecular weight complexes that were soluble and retained function as assessed by glycoprotein gp120 binding activity. CD4 dimers and tetramers by mass were 4 and 15 times as active as CD4 monomer in blocking virus infection with HTLV-III-B in an in vitro cellular assay. The cross-linking chemistry provides an efficient method for producing homomultimers of a glycoprotein.

CD4 (T4) is a surface glycoprotein on certain T-cells and macrophages, which gained particular interest in recent years because of its role as the receptor for human immunodeficiency viruses (1-4). CD4 binds the HIV1 glycoprotein gp120 with a nanomolar dissociation constant (3) and as a consequence this event has created a pathway through which viral infection can occur. The human CD4 gene encodes a protein of 433 amino acids that contains an extracellular domain (residues 1-367), a membrane-spanning region (residues 368-394), and a cytoplasmic domain (residues 395-433; Ref. 5). The extracellular domain shares homology with immunoglobulins and can be further divided into four variable and joining regions (6). Sequences involved in gp120 binding have been localized within the first 113 amino acids of the protein (7, 8).

As the HIV receptor, CD4 has been a focal point of many AIDS research programs (see Ref. 4 and references therein). Soluble forms of the protein effectively blocked viral infections that were induced with laboratory HIV strains (9-13) but were 100-1000 times less active toward virus that had been isolated from infected patients (14). The cause for the large potency difference is unknown (14-16). Attempts to increase the antiviral activity of CD4 have focused on generating CD4 moieties that have a higher affinity for gp120 (17), longer half life (18), or are toxic to gp120 expressing cells (19, 20).

One method for improving the potency of a protein is to produce multivalent adducts where cooperative interactions between binding sites increase the apparent affinity of the protein for its ligand. This approach has been successfully applied to CD4 through genetic engineering by fusing the first two immunoglobulin-like domains of CD4 to the μ-chain of IgM and expressing the fusion product as a recombinant protein (17). The IgM-CD4 protein, a pentamer due to the unit structure of IgM, was 1000 times as active as CD4 alone at blocking laboratory HIV. CD4-IgG fusion products also have been generated in a similar manner (17, 18). The dimeric products showed no increase in antiviral activity, but exhibited over a 100-fold longer half life (18). The lack of increase in potency for the dimer may reflect the difficulty in getting the two gp120 binding sites oriented such that they act cooperatively. In general the time required for the genetic process and lack of flexibility in the final products are drawbacks to this approach.

As an alternative to the genetic approach, protein multimers also can be produced by chemical cross-linking; however, the lack of specificity of most commercial cross-linkers hampers their use. In many instances the cross-linkers attack or sterically block functional sites rendering the products inactive. Like genetic fusions, the chemical approach also can create non-productive adducts if the active sites are improperly oriented, but the flexibility of the linker and ability to vary its length provide more freedom in the spacing of the products than genetic approaches can provide. The sugar residues on CD4 provide a unique situation for targeting cross-linking in that (i) the known glycosylation sites, residues 275 and 298 in the primary structure (21), are outside of the gp120 binding domain of CD4 and (ii) chemistries exist that allow selective activation of sugars (22). Here we describe a novel cross-linking technique which is based on this chemistry and apply the method to CD4 to test the role of multivalence on its antiviral activity. The CD4 cross-linking was an efficient process, converting over 60% of the material into functional multimeric adducts. The cross-linking procedure should be applicable to other glycoproteins, providing a convenient method for producing protein multimers.

MATERIALS AND METHODS

RESULTS

Production of Multimeric CD4 by Chemical Cross-linking—Dimers and tetramers of CD4 have been generated chemically

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1 The abbreviations used are: HIV, human immunodeficiency virus; BMH, bismaleimidohexane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.
through the carbohydrate moieties on the protein using a three-step cross-linking procedure. In the first step CD4 was treated with sodium periodate which converts terminal sialic acid residues on the protein into aldehydes. Next the aldehydes were conjugated with cystamine, converting the reactive group into a thiol. Finally the sulfhydryls were used as the target for cross-linking using thiol-based chemistries. Disulfide-linked complexes were generated by air oxidation and nonreducible complexes generated with the bifunctional maleimide BMH. Fig. 1 show crude mixtures of cross-linked products for air-oxidized (lane c) and BMH-cross-linked CD4 (lane i). In both instances cross-linked products account for over 60% of the input protein. While we attempted to drive the extent of the reaction by regulating the final protein concentration during the cross-linking reaction and by regulating BMH concentrations, the maximum level of cross-linking never exceeded 70%. With higher protein concentrations a significant fraction of the product was lost as very high molecular weight complexes.

The BMH-cross-linked CD4 adducts were fractionated by size on a Superose 6 column. The top panel in Fig. 2 shows absorbance values at 280 nm for selected column fractions containing CD4. Over 50% of the protein is shifted from CD4 monomer (fractions 33–36) to higher molecular weight aggregates. The bottom panel shows results when aliquots from samples were analyzed by SDS-PAGE. Peak fractions for monomer (fractions 33–35), dimer (fractions 30 and 31), and tetramer (fractions 28 and 29) are readily apparent. Under the best conditions about 30% of the protein was converted into dimer and 10% into tetramer. No trimers were formed by cross-linking, suggesting that the interactive unit of CD4 may be a dimer.

The ability of the CD4 multimers to block HTLV-IIIB was quantified in a virus replication assay. As shown in Table I, the CD4 dimer was about 4 times as active as monomer on a mass basis, and the tetramer was 15 times as active. On a molar basis these increases correspond to 30% and 60% for the dimer and tetramer, respectively. In addition to dimer and tetramer, higher molecular weight complexes also were recovered from the sizing column and assayed for activity inhibiting infection by HTLV-IIIB. These complexes were no more active than the monomer alone. The cause for the drop in the activity for the monomer larger than tetramer is unknown but may result from steric problems.

**Biochemical Characterization of the Cross-linked Products—**
Because of the inherent complexity in the cross-linking strategy, it was important to evaluate intermediates at each stage of the process for extent and specificity of the modification reactions. Thus samples were collected after oxidation, conjugation, and cross-linking and independently characterized. Aldehyde formation as a result of oxidation was quantitated with [3H]NaBH₄ by treating the protein with the labeled reductant and then determining the moles of tritium incorporated per mol of CD4. As shown in Fig. 3, 3.46 aldehydes/CD4 were detected for the oxidized material. No label was incorporated into CD4 without prior oxidation. For CD4 that was first treated with neuraminidase and then analyzed, incorporation was decreased by 80%, indicating that sialic acids were the primary target of periodate oxidation. Typical batches of CD4 that were used in these studies on average contained 2.5 mol of sialic acid/mol of CD4. Since each sialic acid through oxidation can yield 2 aldehydes, we infer that about 70% of the sialic acids were modified.

Although sugar oxidation itself was unlikely to perturb function, it was possible that amino acid damage might occur,

**TABLE I**

| CD4          | Typical blocking concentration |
|--------------|-------------------------------|
| rs CD4       | 1.7 μg/ml                     | 34 nM                        |
| CD4 dimer    | −0.41 μg/ml                   | 4 nM                         |
| CD4 tetramer | 0.096 μg/ml                   | 0.48 nM                      |

**Fig. 2. Fractionation of CD4 multimers on a Superose 6 column.** Soluble BMH-cross-linked CD4 was chromatographed on a Superose 6 fast protein liquid chromatography gel filtration column and column fractions analyzed for absorbance at 280 nm. Selected column fractions containing CD4 were subjected to SDS-PAGE and the proteins visualized by staining with Coomassie Blue. Lane a, Bethesda Research Laboratories prestained high molecular weight markers. Lanes 24–39 correspond to the specific fraction numbers indicated.
since certain amino acids are susceptible to oxidation. These side products are easily detected by amino acid analysis. When untreated and oxidized CD4 were characterized by amino acid analysis (Table II), there was no clear difference in the compositions, indicating that amino acid oxidation was not a problem. Finally, to confirm directly that oxidation did not block function, the ability of the modified protein to bind gp120 was assessed in an ELISA format in which Immulon-2 plates were coated with gp120, treated with CD4 or oxidized CD4, and then the binding was determined with a reporter system using an OKT4 antibody-horseradish peroxidase conjugate to detect trapped CD4. There was no difference in binding of CD4 or oxidized CD4 to gp120.

The cystamine conjugation step was evaluated colorimetrically with Ellman's reagent, using free thiol content to indicate conjugation. Table III shows the results from an experiment where the number of –SH groups/CD4 was determined as a function of the cystamine concentration. No cystamine was incorporated into CD4 without prior oxidation. With oxidized CD4, a dose-dependent incorporation of cystamine was observed. Under conditions we routinely use, treatment with 20 mM cystamine, 3.32 –SH groups/CD4 were incorporated. The similarity in the numbers that were determined for thiol groups and aldehydes suggests that all of the aldehydes are modified during the conjugation step. The large excess of cystamine, in addition to driving the amination reaction, serves to block the aldehydes from attacking lysine residues on adjacent CD4 molecules, which otherwise would be a favorable reaction. Similar estimates for the effectiveness of the conjugation step were obtained by reacting oxidized CD4 with [35S]cysteine and quantifying moles of label incorporated per mol of CD4 (data not shown).

Cross-linking of the cystamine-conjugated CD4 was evaluated by SDS-PAGE. The cystamine-conjugated CD4 readily formed multimers when allowed to air-oxidize (Fig. 1, lane c), whereas no complexes were seen with the starting material under the same conditions (Fig. 1, lane b). The disulfide linkage was cleaved with reducing agent, converting the cross-linked adducts back into monomer (Fig. 1, lane e). Lanes f–j show dose-response results for BMH-induced cross-linking. The most efficient cross-linking was achieved with 200 μM BMH. Below 100 μM BMH the cross-linker was limiting. At higher BMH concentrations the formation of monovalent BMH adducts due to an excess of the reagent blocked multimer formation. Cross-linking by BMH is sensitive to protein concentration and free thiol content and had to be titrated for each batch of conjugated CD4. Therefore, large batches of the cystamine conjugate were prepared and stored at −70 °C in appropriately sized aliquots.

The specificity of the periodate-based cross-linking strategy was tested in a series of studies. First, since sialic acid is known to be a primary target of periodate oxidation (22, 30), we tested if the removal of sialic acid with neuraminidase blocked the cross-linking chemistry. Neuraminidase treatment, under conditions that removed approximately 85% of the sialic acid from CD4, resulted in an 80% decrease in the number of aldehydes that were generated (Fig. 3). This number is in close agreement with published results from previous studies (22, 30). For CD4, the decrease in aldehyde formation translated into a similar decrease in cystamine conjugation and finally a decrease in cross-linking. Although the data for CD4 clearly demonstrate that sialic acids are the primary target for periodate oxidation, other sugars can be activated by periodate and thus potentially could be used as sites for cross-linking (31).

Second, the site of cross-linking within the CD4 sequence was localized by CNBr mapping (26). Fig. 4 shows the position of methionines in the CD4 sequence. The four methionines divide the protein into five fragments with apparent masses of 30, 7.5, 7.5, 3, and 5 kDa. CNBr fragment 1 accounts for about two-thirds of the sequence, whereas the other sites dissect the COOH terminus into a series of small fragments. Glycosylation sites at residues 275 and 298 are within CNBr fragments 2 and 3. When CD4 was incubated with CNBr under limiting conditions and cleavage products analyzed by SDS-PAGE (Fig. 4, lane a), 13 fragments were observed. These account for all but two of the possible partial and complete cleavage products. Glycosylated fragments, i.e. those that contain CNBr fragments 2 and/or 3 were identified by analyzing radioactive products released after digestion of [3H]glucosamine-labeled CD4 with CNBr (lane b). Fragment 1-containing products were identified by Western blotting using an antibody against a synthetic peptide from within fragment 1 to probe for immunoreactive cleavage products (lane c). Because of the size and position of fragment 1 within the CD4 sequence, the five fragment 1-containing products form a ladder of products that differ by sequences at the COOH terminus and therefore correspond to fragments 1 + 2 + 3 + 4 + 5, 1 + 2 + 3 + 4, 1 + 2 + 3, 1 + 2, and 1. The sizes of the products were estimated from log (M) versus rt plots of standards that were analyzed on the same gel. These values by difference define the apparent masses of CNBr fragments 2–5 (shown in the schematic at the top of Fig. 4) and in turn were used to predict the fragment compositions of the eight low molecular weight cleavage products (shown at the right of lane e). Lane d shows results obtained when the BMH-cross-linked tetramer was subjected to CNBr mapping. Only fragment 1 was released from the complex, indicating that it is not involved in the cross-linking. Since fragment 1 alone accounts for over two-thirds of the peptide sequence of CD4,
one can infer that the cross-linking is directed at sites near the COOH terminus of CD4. The absence of all other products in the map is consistent with them being cross-linked, but most significant, the absence of fragment 1 + 2 demonstrates directly that fragment 2 is a site of cross-linking, since fragment 1 is not involved. Similar results were obtained for the CD4 dimer (data not shown).

To better characterize the products of the conjugation reaction, we rationalized that if instead of cystamine we incorporated a peptide into ox-CD4, the perturbation would be sufficiently dramatic that we could analyze the products directly. Figs. 5 and 6 show results from such an approach. When CD4 was treated with the peptide GYGKHV-VPNEVVQRLFQVKGR there was a dose-dependent increase in the average molecular weight of CD4 (Fig. 5), indicating that the protein had become modified. At low peptide concentrations there was little effect on the mobility of CD4 but by 1 mM peptide, a significant fraction of the CD4 migrated with an increased apparent molecular weight that is consistent with it containing one peptide/CD4. With 10 mM peptide additional cross-linked species were observed. In addition to the form containing a single peptide, a series of higher molecular weight bands were observed that probably contain two, three, and four peptides/CD4. The need for a large molar excess of the peptide over CD4 to drive the cross-linking reaction is consistent with results obtained for modifying periodate oxidized CD4 with cystamine. The addition of peptide onto the CD4 backbone was confirmed by Western blotting using an antiserum that was raised against the peptide. A prominent immunoreactive band was observed in the sample after cross-linking which is absent from the untreated sample.

The site of conjugate formation was characterized by CNBr mapping. Lanes b and c of Fig. 6 show cleavage products of CD4 and the modified CD4, respectively. For the modified protein a series of reaction products containing zero to four peptides/CD4 were analyzed simultaneously by excising a gel slice that traversed the relevant bands. After treatment with CNBr, the resulting digestion products were electrophoresed orthogonally out of the gel strip into an SDS-polyacrylamide slab gel. From left to right samples run from unmodified CD4 to protein containing four peptide linkages, which creates the curve like appearance of certain cleavage products. As with cross-linked products, fragment 1 was not affected by the modification reaction and therefore appears as a flat horizontal line. On the other hand fragments 1–2, 2–5, and 2–4 are targets as evident by the distortion in their profile. Peptide-containing CNBr fragments were confirmed by Western blotting with the anti-peptide antiserum (lane d).

**FIG. 6.** Localization of cross-linking by CNBr mapping. Parallel samples from the gel analysis shown in lane c of Fig. 5 were subjected to CNBr mapping (26). Duplicate gel strips, each containing CD4 and the series of peptide modified CD4 products, were excised with a razor blade and treated with 30 mg/ml CNBr. Cleavage products were electrophoresed into a 14% polyacrylamide gel and analyzed by silver staining (lane c) or by Western blotting (lane d). The gel strips were oriented from left to right such that they reflect increasing masses and correspond to CD4 alone and products containing one, two, and three peptides, respectively. Lanes a and b show control digests of oxidized (lane a) and untreated CD4 (lane b). The positions of prestained molecular weight markers are indicated at the left of lane a.

**DISCUSSION**

We have demonstrated that the effective concentration of soluble CD4 that inhibits HIV infection can be reduced by converting the protein through cross-linking into chemically linked dimers and tetramers. By mass the CD4 dimers and tetramers were 4 and 15 times, respectively, as potent as the CD4 monomer. The cross-linked adducts were efficiently generated from the soluble product and retained full biological activity as measured by their ability to bind gp120. Unlike conventional cross-linking methods that are hampered by lack of specificity, we have targeted the chemistry at the carbohydrate on CD4 and thus have localized the site of modification away from the functional end of CD4. While other groups (17, 18) have reported similar increases in potency for CD4 protein multimers that were produced genetically by fusing CD4 to immunoglobulins that are naturally occurring multimers, the genetic approach is a lengthy process, limiting the number of products that can be generated and analyzed. Furthermore because of physical constraints that are placed on the fusion molecules, the orientation of the functional groups may be such that they cannot act cooperatively. Cross-linking on the other hand is rapid and can easily be manipulated to change the spacing between the cross-linked products.

The cross-linked CD4 multimers were prepared using a three-step strategy: first, generating reactive aldehydes on the carbohydrate moieties of CD4 in situ with sodium periodate; second, forming cystamine conjugates with the activated CD4 through the aldehydes; and third, inducing cross-linking through the thiol groups on the cystamines either directly by air oxidation or indirectly with the homobifunctional cross-linker BMH. For both sets of products the chemistries were specifically targeted at modifications that were induced on the sugars, leaving the peptide backbone of CD4 unaltered. With this procedure over 60% of the protein was converted

**FIG. 5.** Detection of peptide-CD4 conjugates by SDS-PAGE. Samples of oxidized CD4 containing no addition (lane a), 1 mM peptide (lane b), or 10 mM peptide (lane c) were subjected to SDS-PAGE and either stained with Coomassie Blue (left panel) or subjected to Western blotting (right panel). For protein blots, the peptide containing complexes were stained with rabbit antisera raised to the peptide and visualized with a goat anti-rabbit horseradish peroxidase reporter system. Lane d shows Bethesda Research Laboratories prestained molecular weight markers.
cystamine. Although both reagents were effective, in practice the final product was unreactive to subsequent reactions. We also tested glutathione and cysteine in place of cystamine which was ideal for the strategy we adapted. Only oxidized cystamine was used for the chemistry. When reduced cystamine was used for the cross-linking reaction it was possible that the structural perturbation due to cross-linking might sterically block activity. To rule out this possibility the cross-linked CD4 was tested in an ELISA for the effect of cross-linking on the availability of the critical ORT4A-like epitope and on binding to gp120. In both systems there was no apparent effect of cross-linking on function. Quantitation of the percentage of protein that retained gp120 binding activity revealed that there was no difference in the reactivities of soluble CD4, oxidized product, cystamine-conjugated CD4, or protein after cross-linking. The cross-linking procedure we developed provides an efficient method for producing protein multimers which should be particularly useful for studying systems where multivalent interactions are important for function.

The three-step cross-linking strategy evolved from a series of approaches that themselves proved to be inadequate. First, genetic engineering was used to incorporate a reactive thiol group into CD4 by inserting an unpaired cysteine near the carboxyl terminus of 111- and 180-amino acid constructs of CD4. For unknown reasons the thiol group was unreactive even after taking the protein through a round of reduction and refolding. At best only 0.1 thiols were susceptible to modification, which was impractical for use in subsequent work. Second, using only periodate oxidation and cross-linking through lysines on adjacent protein molecules also yielded poor cross-linking efficiencies. In working with cystamine conjugation, we found that efficient cross-linking required about a 1000-fold excess of the thiolamine to drive the reaction, explaining our inability to run the cross-linking reaction directly. Other approaches have been described where reductive amination was used to couple sugars onto proteins thereby providing a basis for the indirect cross-linking strategy (23, 31); however, in these studies the chemistries were directed at sugars alone in solution and were run in organic solvents. Our study is the first instance where this type of approach has been used to generate homoprotein-protein multimers.

Thiol-based chemistries are rather unique among cross-linking strategies because of the specificity of the reaction and the stability of cross-linkers in aqueous solvents. The use of a low molecular weight reagent such as cystamine, which can be used at high concentrations to drive the conjugation, was ideal for the strategy we adapted. Only oxidized cystamine could be used for the chemistry. When reduced cystamine was employed the final product was unreactive to subsequent reactions. We also tested glutathione and cysteine in place of cystamine. Although both reagents were effective, in practice the cystamine proved to be superior. Similar cross-linking successes were obtained with other glycoproteins that we tested as well, indicating that the process should be directly applicable to a variety of problems where multivalent interactions are important for function.

Although the periodate-based chemistry is specific for carbohydrate on CD4, which itself is not required for function, it was possible that the structural perturbation due to cross-linking might sterically block activity. To rule out this possibility the cross-linked CD4 was tested in an ELISA for the effect of cross-linking on the availability of the critical ORT4A-like epitope and on binding to gp120. In both systems there was no apparent effect of cross-linking on function. Quantitation of the percentage of protein that retained gp120 binding activity revealed that there was no difference in the reactivities of soluble CD4, oxidized product, cystamine-conjugated CD4, or protein after cross-linking. The cross-linking procedure we developed provides an efficient method for producing protein multimers which should be particularly useful for studying systems where multivalent interactions are important for function.

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Production of Multimeric Forms of CD4

SUPPLEMENTAL MATERIAL TO PRODUCTION OF MULTIMERIC FORMS OF CD4 THROUGH A SUGAR-BASED CROSS-LINKING STRATEGY

by Ling Ling Chen, Joseph J. Rosa, Gerat Turner, and T. Lisha Pepinsky

Materials and Methods

Monomeric recombinant CD4 and gold were obtained from BioDex Inc. Cystamine dihydrochloride, sodium periodate, and sodium cyanoborohydride were obtained from Aldrich Chemical Company, Inc. Glutathione, DL-Dithiothreitol (DTT), and 1.2 N acetic acid were obtained from Sigma Chemical Company. The antibody was produced in rabbits against the synthetic peptide GYGKHWPNEWQRLFQVKCRR. The antibody was purified and calibrated against a standard DTNB and after 5 min the absorbance at 410 nm was measured. Samples were calibrated against a standard curve developed with reduced glutathione.

Cystamine dihydrochloride was produced in Chinese hamster ovary cells. The protein, corresponding to amino acids 1-201 in the primary sequence of CD4, was extracted by solubilizing the cells in the presence of 1 M NaOH and 10 mM sodium periodate for 40 min at 23°C and then dialyzed at 4°C against 100 mM sodium acetate pH 5.0. The samples were assayed for protein by absorbance at 280 nm and for free thiols with Ellman's reagent.

Cystamine dihydrochloride was oxidized with sodium periodate for 40 min at 23°C and then dialyzed against 100 mM sodium acetate pH 5.0. The samples were assayed for protein by absorbance at 280 nm and for free thiols with Ellman's reagent. The samples were concentrated 5-fold in centricon-10 units and reduced by adding 2 ml P60G column that had been washed and equilibrated in 100 mM sodium cyanoborohydride. Aliquots from each reaction were dissolved in electrophoresis sample buffer and subjected to SDS-PAGE. Samples were subjected to electrophoresis and transferred to a nitrocellulose membrane. The membrane was stained with Coomassie brilliant blue. A densitometer was used to measure the relative amount of CD4 at each time point.

Effect of cystamine concentration on conjugate formation

C4-CD4(CYT) (1.4 mg) in 10 mM PBS pH 6.5, 5 mM MnCl2 was incubated overnight at 23°C with cystamine-NH2 at the concentrations indicated. The samples were treated with 40 U of PEG column that was equilibrated in 100 mM sodium acetate pH 5.0 and reduced by adding 2 ml P60G column that had been washed and equilibrated in 100 mM sodium cyanoborohydride. Aliquots from each reaction were dialyzed against 100 mM sodium acetate pH 5.0, 2.5 ml of the product was incubated with 40 U of PEG column that had been washed and equilibrated in 100 mM sodium cyanoborohydride. Aliquots from each reaction were dissolved in electrophoresis sample buffer and subjected to SDS-PAGE. Samples were subjected to electrophoresis and transferred to a nitrocellulose membrane. The membrane was stained with Coomassie brilliant blue. A densitometer was used to measure the relative amount of CD4 at each time point.

Cross-linking of CD4 cystamine conjugates—Multimeric forms of CD4-cystamine were generated by treating the samples with the appropriate concentrations of cystamine dihydrochloride. The cystamine-dihydrochloride-treated CD4 (1 mg/ml) was diluted 1:10 with 50 mM MES pH 6.5. The concentration of CD4 in the presence of 50 mM MES pH 6.5 and 5 mM sodium cyanoborohydride was determined by absorbance at 280 nm. In these instances the samples were incubated at 23°C for 30 min and then dialyzed against 100 mM sodium acetate pH 5.0. The samples were assayed for protein by absorbance at 280 nm and for free thiols with Ellman's reagent.

Table I: Effect of cystamine concentration on conjugate formation

| Cystamine-NH2 | CD4/CYT | SH/CD4-CYT |
|---------------|---------|------------|
| 0 mg | 1.0 | 0.0 |
| 1 mg | 0.8 | 0.2 |
| 2 mg | 0.7 | 0.3 |
| 3 mg | 0.6 | 0.4 |

Preparation of neutral alumina-treated C4 (0.3 mg) was treated against 50 mM sodium acetate pH 5.0, 0.3 mg was treated against 50 mM sodium acetate pH 5.0, 0.3 mg was treated against 50 mM sodium acetate pH 5.0, 0.3 mg was treated against 50 mM sodium acetate pH 5.0. During the digestion the sample was continuously mixed in a Thermomixer Block. The neutral alumina-treated CD4 was dialyzed overnight against 100 mM sodium acetate pH 5.0. The samples were assayed for protein by absorbance at 280 nm and for free thiols with Ellman's reagent. The samples were assayed for protein by absorbance at 280 nm and for free thiols with Ellman's reagent. The samples were assayed for protein by absorbance at 280 nm and for free thiols with Ellman's reagent. The samples were assayed for protein by absorbance at 280 nm and for free thiols with Ellman's reagent. The samples were assayed for protein by absorbance at 280 nm and for free thiols with Ellman's reagent.
Production of Multimeric Forms of CD4

| AMINO ACID | PREDICTED COMPOSITION | CONTROL CD4 | OXIDIZED CD4 | OPDM-TREATED CD4 |
|------------|------------------------|-------------|--------------|-------------------|
| Aspartic acid | 30                     | 31          | 31           | 31                |
| Threonine  | 35                     | 26          | 26           | 26                |
| Serine     | 25                     | 33          | 33           | 33                |
| Glutamic acid | 32                     | 34          | 34           | 34                |
| Glycine    | 23                     | 24          | 24           | 24                |
| Alanine    | 17                     | 17          | 17           | 17                |
| Valine     | 20                     | 20          | 20           | 20                |
| Isoleucine | 14                     | 15          | 15           | 15                |
| Leucine    | 13                     | 13          | 13           | 13                |
| Proline    | 9                      | 8           | 8            | 8                 |
| Tyrosine   | 7                      | 6.9         | 6.9          | 6.7               |
| Phenylalanine | 10                    | 10          | 10           | 10                |
| Histidine  | 4                      | 4.1         | 4.1          | 4.1               |
| Lysine     | 38                     | 37          | 37           | 37                |
| Arginine   | 6                      | 8.5         | 8.5          | 8.4               |

*Values shown are moles of amino acid per mole of protein. Theoretical numbers are based on the deduced CD4 amino acid sequence from cloning. Abnormally high values for tyrosine are from glucosamine, which is generated during the hydrolysis and coelutes with tyrosine. Low values for valine and isoleucine result from Ile-Ile, Val-Val, and Ile-Val bonds in the CD4 sequence, which are known to hydrolyze slowly and therefore are reduced in a 24 h hydrolysate (25).