EQUIVALENCE OF HUMAN AND MOUSE CD4 IN ENHANCING ANTIGEN RESPONSES BY A MOUSE CLASS II-RESTRICTED T CELL HYBRIDOMA

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The CD4 glycoprotein is expressed on the surface of mature T lymphocytes that recognize foreign peptide antigens bound to class II MHC molecules. CD4 has been shown to play an important role in stimulating T cell responses to antigen, most likely by acting as an adhesion molecule and by enhancing signal transduction (reviewed in references 1, 2). There is strong evidence that CD4 is a receptor for a relatively conserved region on class II proteins (3–6). We have previously shown that expression of a transfected mouse CD4 (mCD4)[1] construct markedly enhances the IL-2 response of a beef insulin-specific mouse T helper cell hybridoma to beef insulin and results in a new response to pork insulin (7). We have now examined the potential interactions between human CD4 (hCD4) and mouse class II molecules by transfection of a hCD4 construct into this mouse hybridoma. We find that hCD4 is as effective as mCD4 in stimulating responses to beef and pork insulin presented by mouse class II molecules on transfected mouse L cells. These responses are specific for hCD4, since they are blocked by specific mAbs and by the gp120 envelope glycoprotein from HIV-1. These data imply that hCD4 can interact functionally with mouse class II MHC molecules. This finding further distinguishes the interaction between gp120 and CD4 from that between mouse class II molecules and CD4, since gp120 only binds to hCD4 (8) and not mCD4 (9), while mouse class II molecules interact with CD4 from either species.

Materials and Methods

Reagents. Beef and pork insulin were purchased from Sigma Chemical Co. (St. Louis, MO). G418 was purchased from Gibco Laboratories (Grand Island, NY). FITC-conjugated anti-Leu-3a and phycoerythrin (PE)-conjugated anti-L3T4 mAbs were obtained from Becton

This work was supported by National Institutes of Health grants CA-46507, AI-25274, and GM-34991 to J. R. Parnes. P. von Hoegen was supported by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft. M. C. Miceli was supported by a postdoctoral training grant from NIH and by a postdoctoral fellowship from the Arthritis Foundation. B. Tourvieille was supported by a postdoctoral fellowship from the Multiple Sclerosis Society. J. R. Parnes is an Established Investigator of the American Heart Association. B. Tourvieille's present address is Institut Pasteur, B. P. 245, 59019 Lille Cedex, France.

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[1] *Abbreviations used in this paper:* h, human; m, mouse; PE, phycoerythrin; s, soluble; TR, Texas red.

Dickinson & Co. (Mountain View, CA). mAb F23.1 (10), which is specific for Vβ8, binds to the β chain of the beef insulin-specific TCR of BI-141. mAb H10-2.16 (11) is specific for the MHC restriction element I-A^k recognized by BI-141 (12). Both mAbs were used as culture supernatant. mAb GK1.5, which is specific for mouse CD4 (L3T4), was obtained from American Type Culture Collection (Rockville, MD) (13). Purified OKT4B and OKT4D mAbs were a gift from Ortho Diagnostic Systems Inc. (Raritan, NJ) and anti-Leu-3a was a gift from Becton Dickinson & Co. As a secondary stage antibody Texas red (TR)-conjugated rabbit anti-mouse Ig from Axell (Accurate Chemical & Scientific Corp., Westbury, NY) was used. Recombinant soluble CD4 (sCD4) (14) and recombinant gp120 of HIV-1 (15) were a gift from Genentech Inc. (South San Francisco, CA). Highly purified recombinant human IL-2 from *E. coli* was a gift from Cetus Corp. (Emeryville, CA) and had a specific activity of 18×10^6 IU/mg.

Cell Lines. The beef insulin-specific T cell hybridoma BI-141 (7, 12) was cultured in complete RPMI 1640 (Gibco Laboratories). FT5.7 is a transfected line of mouse L cells expressing cell surface mouse MHC class II $A_\alpha{}^bA_\beta{}^k$ molecules (gift from R. Germain) and was grown in complete DME. The IL-2-dependent T cell line HT-2 (16) was maintained in complete RPMI containing 10 IU/ml recombinant human IL-2. Complete culture medium consisted of RPMI 1640 or DME (Gibco Laboratories) supplemented with 1 mM sodium pyruvate, 10 mM Hepes (Applied Scientific, San Francisco, CA), 2 mM L-glutamine (Irvine Scientific, Santa Ana, CA), penicillin (100 U/ml), streptomycin (100 μg/ml) (Flow Laboratories Inc, McLean, VA) and 10% heat-inactivated FCS (Gemini Bio Products Inc., Calabasas, CA).

Expression of CD4 Genes in BI-141 Cells. BI-141 T cell hybridoma cells were transfected by electroporation as described (17) with 3,500 V/cm using a Zapper ZA 1000 (Prototype Design Services, Inc., Madison, WI) with one of two expression vectors, either pSFSVneo (7) or pHβAPr-2-neo (18) containing a full-length cDNA encoding either human CD4 (19) or mouse CD4 (20). Both vectors contain the bacterial gene conferring resistance to neomycin, so transfectants were selected by growth in the presence of 1.5 mg/ml (active concentration) of the antibiotic G418.

Fluorescence Analysis. G418-resistant lines of transfected BI-141 cells were analyzed for cell surface expression of hCD4, mCD4, and TCR. Cells were stained at 4°C with F23.1 culture supernatant for 30 min, washed twice, incubated with 1:10 diluted TR-conjugated rabbit anti-mouse Ig at 4°C for an additional 30 min, washed twice again, and incubated with 1:10 diluted FITC-conjugated anti-Leu-3a or PE-conjugated anti-L3T4 mAbs. 10^4 cells were analyzed with a FACS (Becton Dickinson & Co.) and dead cells were gated out of analysis.

Functional Analysis of BI-141 Transfectants. FT5.7 cells were plated out at a concentration of 10^5 cells/well in a 96-well flat-bottomed plate (Linbro; Flow Laboratories Inc., McLean, VA) and incubated overnight with serial dilutions of beef or pork insulin in complete culture medium. Free insulin was removed by washing with culture medium, and 10^5 responder cells were added per well. Triplicate cultures were incubated in a final volume of 200 μl at 37°C. After 24 h 100 μl of supernatant from each well were harvested and frozen. The thawed supernatants were assayed for the presence of IL-2 by their ability to support the proliferation of the IL-2-dependent line HT-2 (16). Proliferation was assessed by the incorporation of [^{3}H]thymidine (Amersham Corp., Arlington Heights, IL) during an 8-h pulse. Serial dilutions of recombinant human IL-2 were used as a standard. The maximal response correlated to 7.2 IU/ml IL-2.

Inhibition Studies. For inhibition experiments with BI-141 control and transfected cells, purified mAbs, recombinant gp120 or sCD4 were added at the onset of cultures at indicated concentrations. At the concentrations used these reagents did not have any effects on the growth of the indicator cell line HT-2.

Results

BI-141 Transfectants Express Comparable Levels of mCD4 and hCD4. The beef insulin-specific mouse T helper hybridoma BI-141 (12) lacks cell surface expression of mCD4 and uses a Vβ8 TCR to respond to the antigen beef insulin in association

with the mouse class II MHC molecule $A_\alpha{}^bA_\beta{}^k$. We have transfected this hybridoma with full-length cDNA clones encoding either hCD4 or mCD4 in an expression vector. Transfectants expressing CD4 were then subcloned and analyzed for cell surface expression of human or mouse CD4 and Vβ8 (Fig. 1). Vβ8 expression was comparable in non-transfected and in hCD4- or mCD4-transfected cells (Fig. 1). Staining for hCD4 was somewhat lower than that for mCD4, although this may be a reflection of antibody affinity. The level of hCD4 expression was equal to (BI-T4.D3) or slightly lower than (BI-T4.D1) that seen with the same mAbs on human peripheral blood $CD4^+$ cells, while levels of mCD4 expression were slightly higher than on mouse splenic $CD4^+$ cells (data not shown). Transfectants expressing hCD4 were also examined for potential induction of mCD4 expression, but they failed to stain with mAb GK1.5 (13), specific for mCD4.

hCD4 Enhances the Antigen-specific IL-2 Production of BI-141 Cells. Functional analysis of the hCD4-transfected T cell lines revealed a dramatic increase in IL-2 secretion in response to beef insulin presented by $A_\alpha{}^bA_\beta{}^k$-transfected L cells as compared with the parental BI-141 line (Fig. 2 *a*). A new reactivity to pork insulin was also seen in the hCD4-transfected T cells (Fig. 2 *b*). For both antigens the enhanced re-

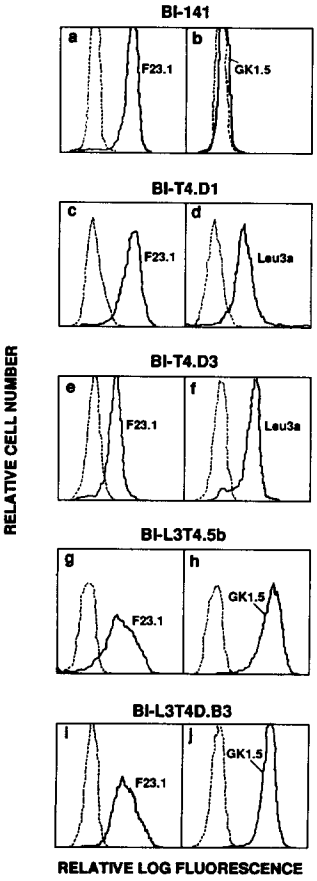

FIGURE 1. Flow cytometric analysis of TCR, hCD4, and mCD4 on BI-141 transfectants. The untransfected parental line, BI-141 (*a*, *b*) and mCD4 transfectants, BI-L3T4.5b (*g*, *h*), and BI-L3T4D.B3 (*i*, *j*) were stained with anti-TCR (F23.1) (*a*, *g*, *i*) and anti-mCD4 (GK1.5) (*b*, *h*, *j*) mAbs. hCD4 transfectants BI-T4.D1 (*c*, *d*) and BI-T4.D3 (*e*, *f*) were stained with anti-TCR (F23.1) (*c*, *e*) and anti-hCD4 (Leu-3a) (*d*, *f*) mAbs.

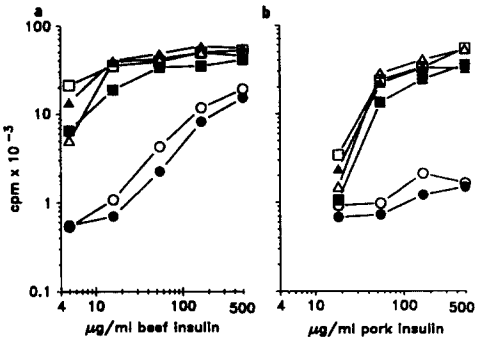

FIGURE 2. Expression of hCD4 increases the response of BI-141 cells to antigen on mouse APCs. T hybridoma cells (10^5) were cocultured with 10^5 FT5.7 APCs pulsed with varying concentrations of beef (*a*) or pork (*b*) insulin. BI-141 ($CD4^-$) (○ and ●), BI-T4.D3 ($hCD4^+$) (□), BI-T4.D1 ($hCD4^+$) (■), BI-L3T4.5b ($mCD4^+$) (△) and BI-L3T4D.B3 ($mCD4^+$) (▲).

sponsiveness of T hybridoma lines expressing hCD4 was indistinguishable from that of transfectants expressing mCD4 (Fig. 2). Similar results were found when antigen was presented by irradiated spleen cells of (B10 × B10.BR)F_1 mice, which also express the appropriate class II MHC molecule (data not shown).

Specific Blocking of hCD4 Transfectants by mAbs. To prove that the enhanced responsiveness of the hCD4 transfectants was a result of functional interactions involving hCD4, we examined the effects of treatment of the transfectants with mAbs specific for hCD4. As shown in Figs. 3 and 4, antigen responses by transfectants expressing hCD4 could be inhibited by mAbs specific for hCD4 (OKT4B and OKT4D), but not by mAb GK1.5 specific for mCD4. Anti-hCD4 mAb Leu-3a was also found to

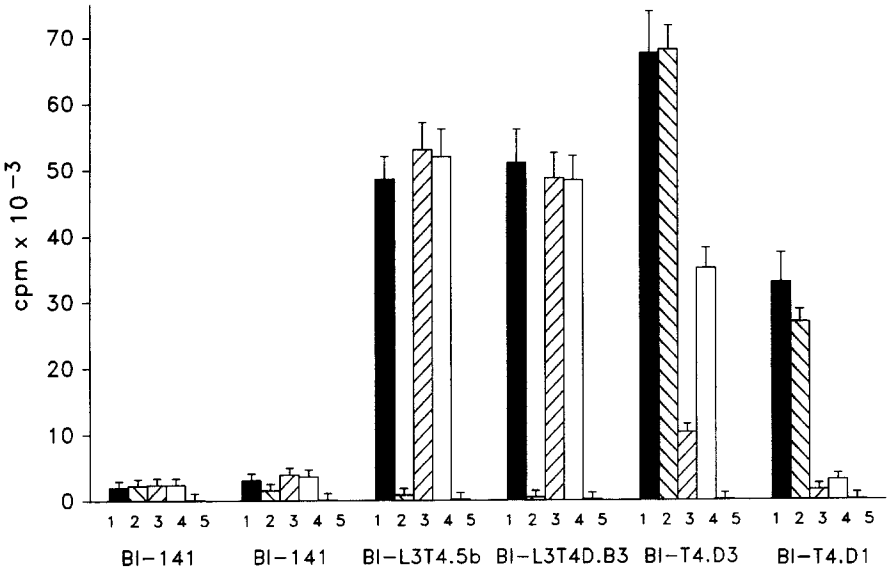

FIGURE 3. Anti-CD4 mAbs specifically inhibit antigen responses by $CD4^+$ transfected BI-141 cells. T hybridoma cells were stimulated with FT5.7 cells pulsed with 100 μg/ml beef insulin. For each cell line IL-2 release is shown for cultures containing either no antibody (lane *1*) or 100 ng/ml of anti-mCD4 (GK1.5) (lane *2*), anti-hCD4 (OKT4B) (lane *3*), anti-hCD4 (OKT4D) (lane *4*), or anti-$I\text{-}A^k$ (H10-2.16) (lane *5*) mAbs. Incubation conditions and IL-2 release assays were as described in Materials and Methods.

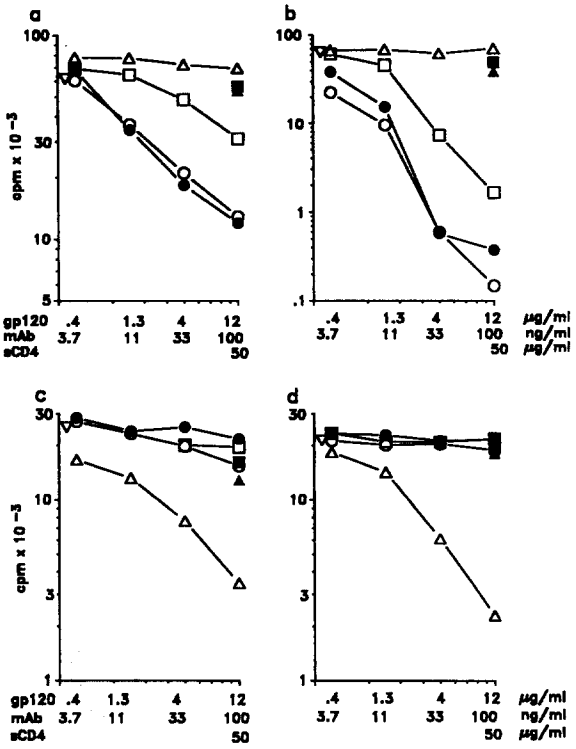

FIGURE 4. HIV-1 envelope glycoprotein gp120 inhibits the IL-2 production of BI-141 cells expressing hCD4. BI-T4.D3 (*a*, *b*) and BI-L3T4D.B3 (*c*, *d*) cells were analyzed for their response against FT5.7 cells pre-incubated with either 100 µg/ml beef insulin (*a*, *c*) (▽) or 500 µg/ml pork insulin (*b*, *d*) (▽). Inhibition was performed with several dilutions of recombinant gp120 (○); mAbs OKT4B (●), OKT4D (□), or GK1.5 (△); or sCD4 (■) at the indicated concentrations. sCD4 (50 µg/ml) was also analyzed in combination with gp120 (12 µg/ml) (▲). IL-2 assay conditions were as described in Materials and Methods.

inhibit responses by the hCD4 transfectants (data not shown). In contrast, transfectants expressing mCD4 could only be inhibited by mAb GK1.5, and not by mAbs specific for hCD4 (Figs. 3 and 4). In all cases complete inhibition of antigen responses was observed in the presence of mAb H10-2.16 (11), which recognizes the $A\beta^k$ polypeptide chain and therefore interferes directly with antigen presentation to BI-141 cells (Fig. 3).

Specific Blocking of hCD4 Transfectants by gp120. We could further demonstrate the specificity of the enhanced responses seen in hCD4 transfectants by taking advantage of the finding that hCD4 serves as the cell surface receptor used by HIV-1 for entry into human cells (8, 21–23). In vitro infection of $CD4^+$ cells by HIV-1 can be inhibited by mAbs specific for hCD4 (21, 22), and hCD4 has been shown to bind to gp120, the exterior envelope glycoprotein of HIV-1 (8). Notably, mCD4 does not bind to gp120 (9) despite its homology to hCD4 (20). Soluble gp120 has been shown to block the hCD4-dependent increase in IL-2 secretion of a human class II–specific mouse T cell hybridoma transfected with hCD4 (24).

We therefore examined whether soluble gp120 similarly inhibits the enhanced responses seen in hCD4-transfected BI-141 cells. As shown in Fig. 4, addition of 12 µg/ml of soluble gp120 completely inhibited the response of the hCD4-transfected lines to pork insulin and blocked the response to beef insulin to a degree comparable to that seen with mAbs specific for hCD4. In contrast, the same concentration of gp120 had no effect upon the mCD4-transfected lines (Fig. 4). As first shown by

Smith et al. (14), genetically engineered soluble forms of hCD4 can bind gp120 and block infection by HIV-1. We therefore examined the effect of soluble recombinant CD4 (sCD4) on the gp120-dependent inhibition of our hCD4 transfectants. The presence of sCD4 abrogated the inhibitory effect of gp120 (Fig. 4). Addition of sCD4 alone did not inhibit IL-2 responses to either beef or pork insulin, suggesting that the affinity of sCD4 for gp120 is much higher than its affinity for cell surface mouse class II molecules.

Discussion

Human and mouse CD4 have each been shown to enhance T cell responses to antigen by interacting with human and mouse class II MHC molecules, respectively (3–7, 25–27). Early studies by Swain et al. (28) first suggested the possibility that hCD4 might be able to interact with mouse class II molecules. They found that human cytotoxic T cells specific for mouse class II molecules expressed hCD4 and were blockable by anti-hCD4 mAb. However, one could argue that this inhibition by anti-hCD4 mAb resulted from steric interference with the TCR, or possibly from transmission of a negative signal to the T cell. Furthermore, any potential interactions between the TCR and hCD4 were strictly syngeneic in the studies of Swain et al. (28). In contrast, the results presented here demonstrate in a direct fashion that addition of hCD4 can enhance mouse T cell responsiveness to peptide antigens bound to mouse class II molecules. These results in no way imply that the converse is true, i.e., that mCD4 can interact with human class II. Indeed binding studies have shown only poor binding between mCD4 transfectants and human B cells expressing class II proteins (29). Studies by Gay et al. (25) and by Sleckman et al. (26) have both previously shown that hCD4 can function in mouse T cells; however, in contrast to our system, the ligand for hCD4 was human and not mouse class II.

We have been unable to detect differences between the effects of mCD4 and hCD4 upon the response of transfected BI-141 cells to either beef or pork insulin presented by mouse class II molecules. In contrast, gp120 only binds to hCD4 and not mCD4, indicating that the binding site on CD4 for class II MHC proteins must be distinct from that for gp120. This conclusion is supported by recent studies showing that mutations not only in the gp120 binding site, but also in other regions of the hCD4 protein, affect the ability of hCD4-transfected COS-1 cells to bind human B cells that express class II molecules (29).

Summary

We have examined the ability of hCD4 to interact functionally with mouse class II MHC molecules using the mouse T cell hybridoma BI-141, specific for beef insulin. We have previously shown that expression of mouse CD4 results in a marked enhancement of IL-2 release by BI-141 cells in response to beef insulin or, in a cross-reactive response, to pork insulin, on the appropriate mouse APCs. We now demonstrate that expression of hCD4 results in an equivalent stimulation of antigen responses by this mouse T cell hybridoma. The specificity of this effect was demonstrated by mAb and gp120 blocking studies. These data provide the first direct evidence for function of hCD4 and in an exclusively mouse system.

We are grateful to Angelika Reske-Kunz and Wolfgang Ballhausen for the BI-141 T cell hybridoma, Ronald Germain for $A_\alpha^b A_\beta^k$-transfected L cells, Tim Mossman for HT-2 cells, Genentech Inc. for soluble gp120 and soluble CD4, Becton Dickinson & Co. for purified Leu-3a mAb, Patricia Rao for purified OKT4B and OKT4D mAbs, and Cetus Corp. for recombinant human IL-2 (30, 31).

Received for publication 31 July 1989.

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