Brief Definitive Report

ACQUISITION OF AN ADDITIONAL ANTIGEN SPECIFICITY AFTER MOUSE CD4 GENE TRANSFER INTO A T HELPER HYBRIDOMA

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Functional T cell subsets expressing CD4 generally recognize antigen (Ag) in the context of class II molecules of the MHC (1). The specificity of Ag-MHC recognition has been demonstrated to be mediated by a clonotypic TCR-α/β complex on the surface of T cells (2), but it has been proposed that other less variable molecules of the Ig gene superfamily, such as CD4, may play an accessory function in receptor-mediated functions. It has been suggested that the variable region–like domains of CD4 bind to nonpolymorphic epitopes of Ia molecules on APC, thus stabilizing and increasing the affinity between the TCR and the Ag-Ia complex (3).

We have previously described the generation of a panel of beef insulin (BI)-reactive T helper hybridomas (4). One group of these hybridomas expressed the CD4 molecule and showed, in addition to its primary beef insulin specificity, a reactivity to pork insulin (PI). A second group of these T helper hybridomas was found to be $CD4^-$ and not to possess reactivity to PI. In the current study, we have used one of the $CD4^-$ clones as a recipient cell for CD4 gene transfer. Transfectants that expressed CD4 on the surface gained PI reactivity and exhibited increased sensitivity for BI. Furthermore, the PI response could be inhibited by the addition of anti-CD4 antibodies, while the augmented response to BI was reduced to the level of response of the recipient cells. These findings suggest that Ag fine specificity does not depend solely on the primary sequence of the TCR, but also may be influenced by the presence of accessory molecules such as CD4. Therefore, the modulation of CP4 may provide an additional mechanism for altering the repertoire of the T cell population.

This work was supported by the Medical Research Council, the National Cancer Institute, and the Natural Sciences and Engineering Research Council of Canada; grant CA-46507 from the National Institutes of Health; and in part by grant SFB 311 from the Deutsche Forschungsgemeinschaft, to A. B. Reske-Kunz. W. G. Ballhausen was supported by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft.

Materials and Methods

Cell Lines. The generation and characterization of BI-specific T cell hybridomas were reported before (4). BI-42.1.83.113.141 (BI-R) represents a $CD4^-$ BI-specific T helper hybridoma derived by three single-cell cloning steps. The T cell line BW5147 was obtained from the American Type Culture Collection, Rockville, MD.

Cytofluorometric Analysis. Cells were treated with 50 μl culture supernatant of clones GK1.5 (1), KJ16-133 (5), and H35-89.9 (6) for 30 min, were washed twice, and were labeled with fluorescein-conjugated goat anti-rat IgG $F(ab')_2$ fragment (Southern Biotech Assoc., AL) at 1/30 dilution for 30 min. Cells were analyzed with an Epics C cell sorter using a three-decade log scale.

T Cell Proliferation Assay. Reactivity was assessed by the amount of IL-2 secreted by 1×10^5 hybrid cells when stimulated with Ag and 2×10^5 irradiated (2,000 rad) (B10 × B10.BR)F_1 spleen cells for 24 h. 50 μl of cell-free supernatant was added to 2.5×10^3/50 μl IL-2-dependent CTLL indicator cells as described in reference 7. For inhibition experiments with anti-CD4, mAb GK1.5 ascites was present at 1:900 dilution. The anti-$RT1A^a$ MHC class I rat IgG2b mAb ascites YR1/100 (Serotec, Blackthorn, United Kingdom) was used at similar concentrations in inhibition control experiments.

Expression Vector. A full-length mouse CD4 cDNA clone was constructed using the combination of cDNA clones pcL3T4-14 and pcL3T4-C7 (8). The coding sequence flanked by 118 nucleotides of 5′ untranslated region and 4 nucleotides of 3′ untranslated region was introduced by blunt-end ligation at the unique Xho I site of the pSFSVneo vector, (a gift from P. Berg, Stanford, CA) and the SFFVp502 clone, described in reference 9.

Northern Blot Analysis. RNA work was carried out according to standard procedures (10). 10 μg of RNA was simultaneously hybridized with nick-translated cDNA clone p3C (a gift of D. Littman, San Francisco, CA) and a β-tubulin probe.

Transfection. For transfection by electroporation, 30 μg of linearized pSFSVn-LC7 DNA was added to 2×10^6 BI-R cells, followed by six pulses of 5 μs with 8 kV/cm. Cells were seeded at a density of 2×10^5/ml into microwell plates for G418 selection. On day 21, G418-resistant colonies were analyzed by indirect GK1.5 surface staining.

Results and Discussion

Our experiments were based on the observation that T helper hybridomas established from BI-primed lymph node T cells of (B10 × B10.BR)F_1 mice (4) could be divided into one group expressing CD4 and specific for BI and PI, whereas a second group of hybridomas was $CD4^-$ and was only reactive with BI but not with PI. Therefore, we decided to examine whether a BI-specific hybridoma of the $CD4^-$ group, represented by clone BI-42.1.83.113.141 (BI-R), would acquire PI reactivity after CD4 gene transfer. For the transfection study, we used a full-length cDNA of mouse CD4 molecule (8) and the SFF V-LTR (10) (see Fig. 1). After transfection, G418-resistant clones were tested for their reactivity towards PI in the presence of syngeneic splenic APC. The clone with the highest reactivity towards PI, BI-42.1.83.113.141-30.3-LC7.67 (BI-T), was analyzed in more detail. The cytofluorometric data obtained using a mAb against mouse CD4, GK1.5 (1), demonstrated that the recipient hybridoma BI-R did not express detectable amounts of cell surface CD4 (Fig. 2). GK1.5 staining of the transfected clone BI-T resulted in an intense fluorescence signal, indicating that this cell has now gained the ability to display CD4 on its surface (Fig. 2). However, indirect surface staining of both the recipient and transfected cell with the anti-TCR mAb KJ16-133 (5) and anti-LFA mAb H35-89.9 (6) demonstrated that the number of TCR complexes and LFA molecules accessible on the transfected cell was not affected by the surface expression of the CD4 gene product (Fig. 2).

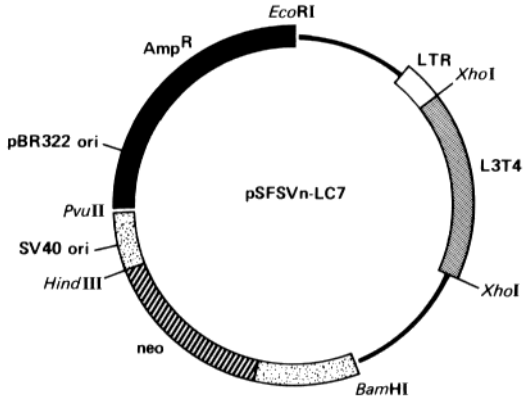

FIGURE 1. Structure of the pSFSVn-LC7 expression vector. A full-length mouse CD4 cDNA was cloned into the pSFSVneo vector, derived from pSV2neo (9) (large Eco RI-Bam HI fragment) and the SFFVp502 clone (10).

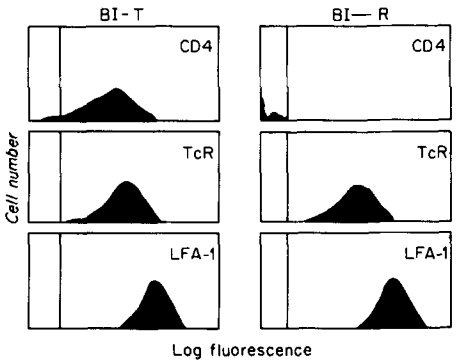

FIGURE 2. Cytofluorometric analysis of recipient and transfected cells with mAb GK1.5 (CD4) (1), mAb KJ16-133 (TCR) (5), and mAb H35-89.9 (LFA-1) (6). The vertical lines denote the position of the gate set to exclude nonspecific fluorescence obtained in the controls stained solely with the secondary antibody.

Northern blot analysis of the cells indicate that the recipient clone, BI-R, did not express detectable levels of CD4 message (Fig. 3, lane *2*). However, 3.2-kb CD4-specific transcripts were detected in RNA preparations from the transfected clone, BI-T (Fig. 3, lane *1*). RNA of the $CD4^+$ clone BI-42.1 (4) (Fig. 3, lane *3*) and of mouse thymoma line BW5147 (Fig. 3, lane *4*) were used as positive and negative controls, respectively.

We studied the effect of CD4 surface expression on the Ag recognition pattern of BI-T and BI-R by assaying for the production of IL-2 after stimulation with PI or BI in the presence of splenic $H\text{-}2^{bxk}$ APC (see Fig. 4). Earlier studies of the response pattern of a panel of BI/PI-specific T helper hybridomas revealed that for the activation of an individual clone ~15-fold more PI than BI was needed to induce the same level of response (4). The recipient cell, BI-R, showed a considerable response to BI, which required a 50-fold higher concentration of BI than the response of BI-T cells. However, PI in a concentration of up to 640 μg/ml failed to induce IL-2 production in the recipient cell line (Fig. 4 *a*). On the other hand, the transfected clone, BI-T, showed in addition to an increased BI response, a strong PI reactivity (Fig. 4 *a*). To confirm that the PI specificity was due to CD4 surface expression, we performed the proliferation assay in the presence of the rat IgG2b anti–mouse CD4 mAb GK1.5. The results of this inhibition assay, depicted in Fig. 4 *b*, clearly demonstrate that the T cell response to PI could be completely abolished by anti-CD4 mAbs (Fig.

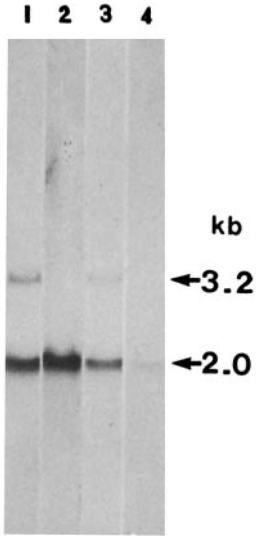

FIGURE 3. Northern blot analysis of CD4 mRNA expression. Lane *1*, RNA from transfected cell BI-T; lane *2*, RNA from recipient cell BI-R; lane *3*, RNA from CD4$^+$ hybridoma BI-42.1 (4); lane *4*, RNA from thymoma BW5147. 2.0-kb beta-tubulin signals indicate amounts of RNA loaded in each lane.

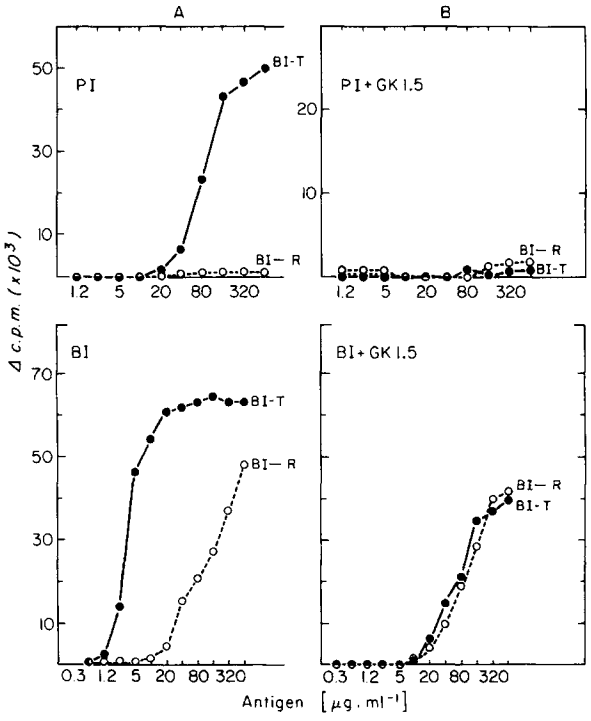

FIGURE 4. Characterization of the Ag-recognition pattern of BI-T and BI-R. (*A*) Response towards BI and PI. (*B*) Response towards BI and PI in the presence of anti-CD4 mAb GK1.5. *(Solid line)* BI-T; *(dashed line)* BI-R. The medium control for BI-T was 1,330 cpm and 720 cpm for BI-R. The nonspecific inhibitory effect on BI and PI responses by the presence of rat IgG2b control mAb YR1/100 directed against rat MHC-class I Ags was determined to be <4.2% in comparison to the unblocked response.

4 *b*) and was therefore strictly dependent on CD4 interactions. BI-induced production of IL-2 by BI-T was reduced by the presence of anti-CD4 mAbs to the level elaborated by the CD4$^-$ recipient BI-R cells in the absence or presence of the mAb (Fig. 4 *b*). Inhibition control experiments with the irrelevant rat IgG2b mAb YR1/100 directed against rat MHC-class I Ags indicated that BI- and PI-induced stimulation decreased only slightly by 2.1–4.2%.

This system enabled us to compare the effect of CD4 on the T cell response directly by examining the activation pattern of the $CD4^-$ helper T cell with the CD4-expressing transfectant of the same cell. In our studies, the $CD4^-$ recipient cell showed an efficient response towards BI; however, the same $CD4^-$ helper T cell was not activated by PI concentrations of up to 640 μg/ml, suggesting that the affinity of the TCR for the PI-Ia complex was too low to generate a potent stimulatory signal in the absence of CD4 (Fig. 4 *a*). It should be noted that all $CD4^-$ BI-specific T cell hybridomas tested thus far lack reactivity to PI and that all PI-reactive T cells do express the CD4 molecule (Reske-Kunz, A., results unpublished). When CD4-transfected cells were stimulated with BI, a markedly increased response was seen, suggesting that even under conditions of effective stimulation, the presence of CD4 could promote stable cellular interactions (Fig. 4 *a*). Of more interest was the result that PI, which is unable to stimulate $CD4^-$ recipient cells, was now able to trigger CD4-expressing transfectants to secrete high levels of IL-2 (Fig. 4 *a*).

Saizawa et al. (11), using anti-CD4 and various anti-TCR mAb as crosslinkers to activate $CD4^+$ cells, suggested that binding of the TCR to an Ag-MHC in the presence of CD4 molecules is more effective for T cell activation. Our data, under more physiological stimulation conditions, demonstrate that the binding of the TCR to the BI-MHC is 50-fold enhanced by the presence of CD4 molecules. The failure of the CD4-dependent Ag PI to activate transfected cells in the presence of anti-CD4 mAb GK1.5 (Fig. 4 *b*) may be due to either steric inhibition or conformational changes in CD4, which prevent it from becoming part of the TCR complex that binds to the PI-MHC.

Sleckman et al. (12) presented data on the transfer of the human CD4 gene into a mouse T cell hybridoma specific for human HLA-DR Ags by retroviral infection. Both the recipient and the CD4-transduced cells produced IL-2 in response to HLA-DR Ags. Our results extend these observations of a role for CD4 in recognition of xenogeneic class II MHC Ags to a syngeneic system of MHC-restricted Ag recognition. Furthermore, the system described here has the advantage compared with that employed by Gay et al. (16) that no potential interactions of the TCR, CD4, and class II MHC molecules will be inhibited because of cross-species differences in these molecules.

Finally, the data presented in this study differ from previous by demonstrating that CD4 is not only capable of enhancing T cell responsiveness to a given Ag, as measured by increased release of IL-2 (3, 12, 13), but that it may also play a critical role for helper T cells enabling them to be triggered by Ags at concentration levels that would have failed to stimulate in the absence of CD4. Therefore, we propose that the stimulatory effect of an Ag for helper T cells does not depend solely on the expression of the clonotypic TCR, but may also be determined by the presence of CD4 on the surface of cells.

Summary

We have transfected the mouse CD4 gene into a beef insulin (BI)-specific murine T helper hybridoma that lacks CD4 surface expression. The CD4-expressing transfectants have acquired an additional reactivity for pork insulin (PI), which was not detectable in the original recipient cell. The transfectants' response to PI can be completely abrogated by anti-CD4 antibodies. The trans-

fected clone showed a 50-fold increased sensitivity towards BI in comparison to the same $CD4^-$ hybridoma. These experiments suggest that CD4 may be important in determining the antigen fine specificity and, therefore, may also play a role in altering the T cell repertoire.

We thank Ms. U. Arndt, U. Bonifas, and H. Broughton for expert technical assistance.

Received for publication 10 November 1987 and in revised form 31 December 1987.

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