Brief Definitive Report

IDENTIFICATION OF THE ALTERNATIVELY SPLICED EXONS OF MURINE CD45 (T200) REQUIRED FOR REACTIVITY WITH B220 AND OTHER T200-RESTRICTED ANTIBODIES

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CD45 (T200, leukocyte common antigen) is a major cell membrane glycoprotein expressed on all hematopoietic cells, except mature erythrocytes. The antigen has been characterized in mouse, rat, human, and chicken and has similar properties in all species (1-5). Structural variants of CD45 that are selectively expressed on specific classes of lymphocytes have been detected by both biochemical and immunological methods. At least four forms of CD45, ranging in size from M_r 180,000 to 220,000, can be resolved by SDS-PAGE, and anti-CD45 mAbs have been obtained that discriminate between the different M_r species. Such restricted CD45 (CD45R) mAbs have proved useful in identifying subpopulations of functional cells that display particular forms of CD45. In the rat, mAb OX-22 reacts with the 220,000 M_r species of CD45 on B cells and also a subfraction of the 190,000, 200,000, and 220,000 M_r species of CD45 from peripheral T cells. This mAb divides rat $CD4^+$ T cells into an OX-22^+ subset that mediates graft-vs.-host activity and an OX-22^- subset that provides B cell help in vitro and in vivo (reviewed in reference 6). In the human, two anti-CD45R mAbs, 2H4 and UCHL-1, have been identified that reciprocally split $CD4^+$ T cells into two distinct subpopulations; $2H4^+$ cells when activated, can induce suppression (7), whereas UCHL-1^+ cells provide B cell help (8). mAbs 2H4 and UCHL-1 selectively react with the 220,000 and 200,000 M_r species (9) and the 180,000 M_r species of CD45, respectively (10). In the mouse, mAbs have been described that recognize a 220,000 M_r form of CD45 referred to as B220 and react either with only the B cell lineage or with B cells and a subpopulation of T cells (11). These antibodies have been used to identify cells of the B lineage, including pre-B cells in the bone marrow.

Recent studies of the molecular genetics of CD45 and the determination of the predicted primary structures of the polypeptides encoded by CD45 cDNAs from a variety of lymphoid tissues and cell lines in the rat (3), mouse (1, 2), and human

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(4, 12) have shown that the protein heterogeneity of the antigen is due to the variable expression of 3 alternatively spliced exons (A, B, and C) proximal to the 5' end of the gene. Each exon encodes sequences of ~50 amino acids that are differentially expressed to generate at least five forms of CD45 (1-4, 12). These data have established the molecular basis of the structural heterogeneity of CD45 and provide the opportunity to more precisely define the antigenic specificities of mAbs that recognize different forms of CD45. mAb OX-22 recognizes an antigenic determinant displayed by two tryptic peptides of rat CD45 derived from a region proximal to the NH_2 terminus of the molecule. Although the NH_2-terminal sequences of both peptides are encoded by exon B, both peptides are sufficiently large to allow the possibility that the OX-22 antigenic determinant is dependent upon the expression of exon C (3). In the human, it has been shown that exon A is required for expression of the 2H4 epitope (13).

In this report, we have prepared four cell lines that express each of the individual forms of recombinant mouse CD45 that have been presently identified in the mouse (1, 2), and we have used them to determine which alternatively spliced exons are required to generate the antigenic determinants recognized by three widely used CD45R mAbs, RA3-6B2, RA3-2C2 (11), and 14.8 (14). In addition, another anti-mouse CD45R mAb, C363.16A, has been characterized that has a novel restricted specificity.

Materials and Methods

mAbs. RA3-6B2, RA3-2C2, and TIB122 (M1/9.3.4.HL.2) were obtained from the American Type Culture Collection, Rockville, MD, and have been characterized previously (11). mAb 14.8 was a gift from Dr. P. Kincade (Oklahoma Medical Research Foundation) (14). mAb C363.16A, an IgG2a, is produced by a hybridoma derived from rat spleen cells immunized with a cloned mouse Th line (Bottomly, K., M. Lugman, L. Greenbaum, S. Carding, J. West, T. Pasqualini, and D. B. Murphy, manuscript submitted for publication). I3/2 is an anti-CD45 mAb and has been characterized previously (15).

cDNA and Retroviral Constructs. A full-length mouse CD45 cDNA containing all three alternatively spliced exons was constructed from a pUC9 subclone of an Eco R1 fragment spanning the 5' coding region derived from λmLC1 and a similarly subcloned Eco R1 fragment from λmLC2 that includes the remaining 3' coding sequence (1). Cla 1 linkers were added at the 5' Nhe 1 site of the first fragment and Xho 1 linkers were added at the 3' Cla 1 site of the second (see Fig. 1). The Cla 1/Eco R1 and Eco R1/Xho 1 fragments were ligated into pAT153/Pvu II/8 modified by the introduction of an Xho 1 site to yield a full-length CD45 construct lacking a poly A addition site. This Cla 1/Xho 1 fragment was blunt end ligated into the Xba 1 cloning site of pARV1 (16). Inserts in the correct orientation were identified by restriction enzyme analysis.

Another form containing alternative exons B and C was obtained from clone B15 kindly provided by Y. Saga (Sloan-Kettering Institute for Cancer Research, New York) (2). A full-length construct was made by the ligation of the B15 cDNA to an Eco RV/Eco R1 fragment and then an Eco R1/Xho 1 fragment of the mouse CD45 cDNA (Fig. 1). This CD45 construct was subcloned into a modified pUC18 vector and subsequently excised from the plasmid using Sal 1 and Xho 1 enzymes.

The other two forms (expressing exon C or no alternative exons) were derived by oligonucleotide site-directed mutagenesis from a 5' 2.2-kb Eco R1/Sma 1 fragment of the 3 exon form of CD45 subcloned into M13mp19 using 2 oligomers (24 and 25 mers). This generated the precise deletions of exons A and B and exons A, B, and C, respectively, which were verified by sequencing and then the fragment was subcloned back into the original vector.

Transfection and Infection. PA12 cells were transiently transfected with each retroviral construct, and after 48 h, the culture supernatant was used to infect Ψ2 cells. These cells were

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then selected in 350 μg/ml of active G418 and individual colonies were isolated and subsequently analyzed.

Flow Cytometry. Analysis of CD45 expression was performed using the Salk Institute flow cytometer, essentially as described in reference 17. Undiluted tissue culture supernatants (100 μl) containing mAbs I3/2, RA3.6B2, RA3.2C2, or C363.16A or a saturating dilution of ascites containing mAb 14.8 were used as the primary reagents to stain 10^6 cells. Then, 50 μl of FITC-labeled goat anti-rat IgG (Organon Teknica-Cappel, Malvern, PA) was used as the second antibody at a dilution of 1:20.

Results and Discussion

The objective of these studies was to map the antigenic determinants recognized by anti-CD45R mAbs more precisely by determining their reactivity with cell lines expressing individual forms of recombinant mouse CD45. Three anti-B220 mAbs, RA3-6B2, RA3-2C2, and 14.8, were chosen for study because of their extensive prior use to distinguish lymphocyte subpopulations. RA3-6B2 appears to be specific for CD45 on B cells, whereas RA3-2C2 and 14.8 recognize a form present on B cells and at lower levels on $CD8^+$ T cells (18, 19). A fourth antibody, C363.16A, reacts with the 190,000 and 220,000 M_r forms, but not the 180,000 M_r form of CD45 and appears to differentiate between two subpopulations of $CD4^+$ T cells (20).

To obtain cell lines expressing individual forms of mouse CD45, four full-length CD45 cDNAs were constructed as described in Materials and Methods, each encoding a single form of CD45 glycoprotein containing different combinations of variable exons A, B, and C. The cDNAs were then ligated into the retroviral vector pARV1 (Fig. 1). These retroviral constructs were transiently transfected into PA12 cells and the supernatants were used to infect Ψ2 cells. Infected Ψ2 cells were selected by their resistance to G418, and individual colonies were isolated and then analyzed by flow cytometry for the expression of mouse CD45 using the mAb I3/2. Cloned cells expressing the highest amounts of CD45 on their cell surface were subsequently used for the analysis of the binding specificities of the CD45R mAbs. The results of these binding studies are shown in Fig. 2. mAbs RA3-2C2 and 14.8 had similar patterns of reactivity, staining only Ψ2 cells expressing the largest form of CD45 with sequences encoded by exons A, B, and C. As the antibodies do not react with the CD45 glycoprotein containing the regions encoded by exons B and C, we can conclude that both these mAbs recognize antigenic determinants that are dependent upon the expression of exon A. In this respect, the mAbs are similar to the 2H4 mAb in the human. However, in contrast to 2H4, which splits the $CD4^+$ T cell subset in the human (9), neither RA3-2C2 nor the 14.8 antibody has been reported to react with $CD4^+$ T cells in the mouse. This may either reflect a species difference in the splicing out of exon A in $CD4^+$ T cells or a difference in the ability of the mAbs to detect their respective antigens.

mAb C363.16A binds only to the forms of CD45 encoded by cDNA constructs containing exon B and, thus, clearly differs in specificity from RA3-2C2 and 14.8 mAbs. This antibody not only reacts with B cells and $CD8^+$ T cells but also splits $CD4^+$ T cells (20). Although the most simple interpretation of the dependence of antigenic determinants upon the expression of a specific alternatively spliced exon is that the exon encodes the polypeptide region to which the antigenic determinant is localized, it cannot be excluded on the basis of the present data that other regions of CD45 influence the antigenic site.

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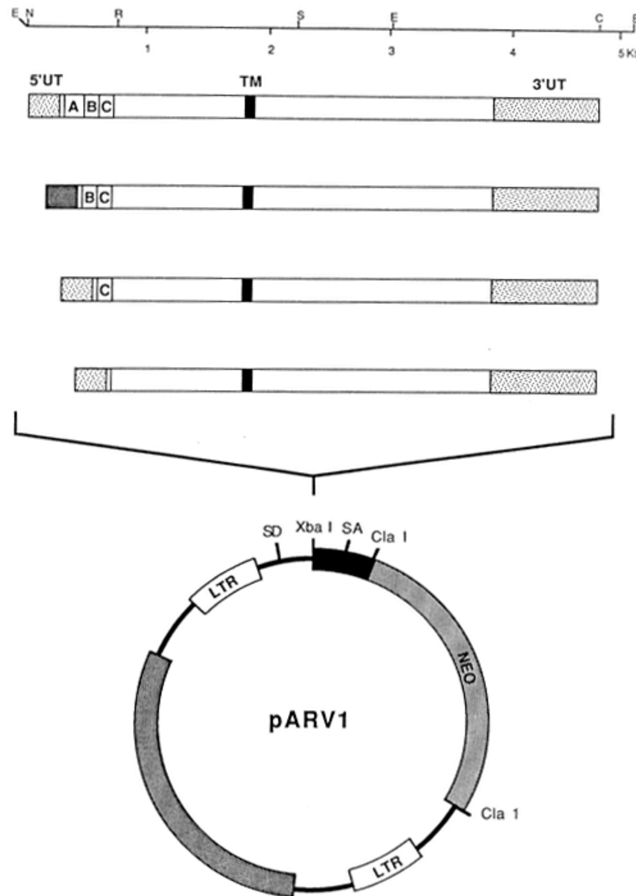

FIGURE 1. Structures of four mouse CD45 constructs that were ligated into the retroviral expression vector, pARV1 at the Xba 1 site, between the splice donor (*SD*) and splice acceptor (*SA*) sites. The shaded region represents the transmembrane region (*TM*) and the stipled boxes, the 5' and 3' untranslated regions (*UT*). *A*, *B*, and *C* represent the three alternatively spliced exons and the second construct (expressing exons B and C) has an alternative 5' untranslated region derived from clone B15 (2). *E*, *N*, *R*, *S*, and *C* represent Eco R1, Nco 1, Eco RV, Sma 1, and Cla 1 restriction sites, respectively. The unmarked shaded region in pARV 1 represents pUC9 sequence conferring Amp^r.

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RA3-6B2 does not bind to any of the four constructs expressed in Ψ2 cells. One explanation of this result could be that the determinant is dependent upon the expression of a combination of alternatively spliced exons not represented by the four constructs that were tested. Another is that the antigenic determinant requires a post-translational modification of the CD45 antigen, such as the addition of specific oligosaccharide moieties that do not occur correctly in Ψ2 cells. Expression of sufficiently high levels of specific forms of CD45 in mutant $CD45^-$ lymphoid cells has not been obtained using the present retroviral construct to directly test this latter hypothesis. However, preliminary data suggests that neither neuraminidase digestion nor mild periodate treatment affects the RA3-6B2 determinant. The antigenic determinants of two widely used B220 antibodies have now been defined and the expression of two of the three alternatively spliced exons can now be monitored by specific antibodies. It should now be possible to establish in which lymphoid cells and at what stages of development these alternative exons are expressed, which may provide insight into their functional significance.

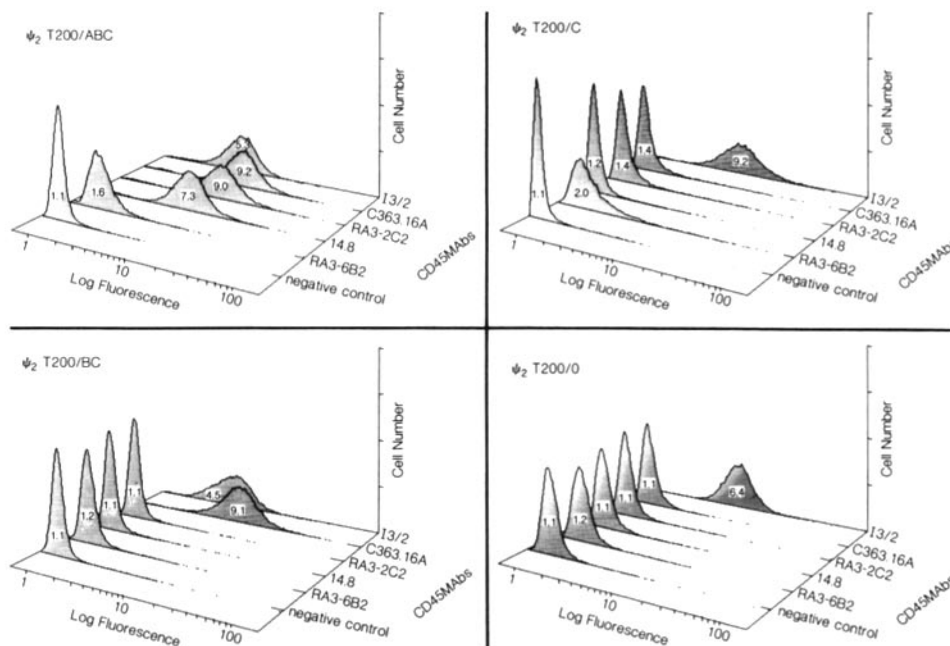

FIGURE 2. Analysis of anti-CD45 antibodies binding to Ψ2 cells expressing different forms of the molecule. Four Ψ2 cell lines were assayed by indirect immunofluorescence using mAbs I3/2, RA3-6B2, RA3-2C2, 14.8, C363.16A, and a negative control, tissue culture medium alone. *A*, *B*, and *C* represent the variable exons that were expressed and T200/0 represents the construct lacking the alternatively spliced exons. The *x*-axis represents fluorescence intensity on a logarithmic scale and the vertical scale is cell number. The mean fluorescence is given in each histogram and the number relates to the intensity of fluorescence over the negative control. The weak labeling of the Ψ2/T200/C cell line with the RA3-6B2 antibody seen in this particular experiment was not reproducible.

Summary

Cell lines expressing specific variants of murine CD45 (T200) were established by infection with retroviral constructs of four cDNAs encoding different forms of CD45. These lines were then used to determine which sequences encoded by alternatively spliced exons of CD45 were required to generate antigenic determinants recognized by anti-CD45 mAbs. The binding of two B220 antibodies (14.8 and RA3-2C2) to CD45 was dependent on the expression of the first alternatively spliced exon (exon A). A third B220 antibody, RA3-6B2, did not bind to any of the forms of CD45 expressed on fibroblasts. A newly defined anti-CD45R antibody, C363.16A, reacts with an antigenic site dependent upon the expression of the second alternatively spliced exon, exon B.

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