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MUTATIONS AFFECTING ANTIGEN PROCESSING IMPAIR CLASS II-RESTRICTED ALLORECOGNITION

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Both exogenously derived and endogenously derived Ag generally require processing for their optimal binding and presentation by class I and class II major histocompatibility proteins. It is not known whether steps involved in Ag processing also affect the recognition of alloreactive T cells. We have recently described B cell mutants which have general defects in the processing and presentation of a variety of exogenous Ag to class II restricted T cells. In this report we have studied the ability of these processing mutants to stimulate a set of anti-DR3-specific alloreactive T cells clones. These processing/presentation mutants express normal MHC class II molecules, both in terms of primary sequence and cell surface abundance, but they appear unable to generate effective peptide-MHC complexes. When tested for their ability to stimulate MHC class II alloreactive T cell clones, only one of four T cell clones was stimulated by these mutants; the other three alloreactive T cell clones were not stimulated by either of two different mutants. Both of these mutants express normal levels of the accessory molecules, LFA-3 and ICAM-1. The inability of these mutants to stimulate three of four alloreactive clones indicates that the capacity to be recognized by many alloreactive T cells is linked to the Ag processing capacity of a stimulator cell.

The MHC class I and class II proteins function as peptide carriers for Ag which are presented to T cells in the form of peptide-MHC complexes. Conventional Ag, with few exceptions, require denaturation and some form of Ag processing by an APC (1–3). The processing of class II-restricted Ag occurs intracellularly in acidic, probably endosomal vesicles (4, 5) and is sensitive to lysomorphotropic agents such as chloroquine (6). The biochemical steps involved in the processing of Ag and the precise structure of antigenic peptides are not well defined, but can be mimicked by synthetic peptides (7, 8). Peptides have been demonstrated to bind to both MHC class I and class II proteins; the phenomenon of MHC restriction is attributable to the binding of peptides by MHC molecules in an allele-specific manner (9–13). Although Ag-specific T cells recognize peptide-MHC complexes, the nature of the recognition of non-MHC identical stimulator cells by allogeneic T cells is unclear. It could involve direct recognition of the polymorphic differences in the foreign MHC molecule or recognition of particular peptide-foreign MHC complexes.

We have recently characterized several B cell mutants which are unable to present intact protein Ag to MHC class II-restricted T cells, although they present antigenic peptides normally (14, 15). The class II genes in these mutants are normal, as is the level of class II expression on the cell surface. Here we have assessed the ability of alloreactive T cells to recognize the MHC class II molecules on the Ag processing and presentation defective mutants. We find that mutations which affect the processing of exogenous Ag also impair the recognition of MHC class II-specific alloreactive T cells.

MATERIALS AND METHODS

B cell lines. The B lymphoblastoid cell lines used and their origins are described in Table I. (14, 16–18). The Ag-processing mutants 9.5.3 and 9.10.3 were selected for loss of a polymorphic determinant defined by monoclonal antibody 16.23 (14). The Ag processing and presentation mutants have wild-type α and β genes (15).

Immunofluorescence analysis. B cell surface markers were analyzed quantitatively on a fluorescence-activated cell sorter (FACSTAR, Becton-Dickinson Diagnostics), with FITC derivatives of antibodies to ICAM-1 (60.3, from Ed Clark, University of Washington) and HLA-DR (VI.15) (16). Cells were indirectly stained for LFA-3 by TS/9.1 (from Alan Krensky, Stanford University) and a goat antibody to mouse IgG (Tago, Inc., Burlingame, CA). Cells were stained in PBS containing 1 mg/ml myoglobulin and washed twice immediately before analysis. A difference of 11 fluorescence channels represents a doubling of intensity.

T cell clones. Four DR3-specific T cell clones were derived from nonparous individuals. One clone, C6, is specific for DR52a molecules and was the kind gift of Eric Mickelson (19). Clones 62D10 and 24F7 were from a DRw6, w6, DRw52a donor, and both were derived from protocols designed to reduce the number of anti-class I specific T cells. Donor T cells (1×10^6) were stimulated in vitro for 4 days with 1×10^5 irradiated class I positive, DR-negative cells (6.1.6 in the case of 62D10 and 9.22.3 for 24F7) and the responding population was treated with an anti-IL-2R antibody (4E3, the kind gift of Steve Dower, Immunex Corp., Seattle, WA) plus C. Surviving cells were placed at 10^6 lymphocytes/ml and stimulated with 10^5 irradiated 8.1.6 cells for 5 days. T cells were cloned as described elsewhere (18, 20), with 10, 4, 2, and 0.5 cells/wells. Both 62D10 and 24F7 were obtained from wells containing 2 cells/wells. Clone 4.26A was from a DR18, 11, DRw52a,b donor. The responder cells were isolated and cloned after a 4-day stimulation by irradiated lymphocytes from a DR17, 11, DRw52a,b donor.

Proliferation assays. DR3-specific alloreactive T cells (10^4) were cocultured with various mitomycin C-treated B cells as stimulators in RPMI 1640 with 10 to 15% heat-inactivated human AB serum. Proliferation was measured by determining the incorporation of [^{3}H] thymidine added for the final 16 to 18 h of a 72-h assay. All values reported are the median value of triplicate samples, from which the background, taken as the sum of the incorporation resulting when both responders and stimulators were incubated separately, has been subtracted.

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TABLE I

Cell lines and expressed class II specificities

Cell Line	Progenitor	Haplotype 1	Haplotype 2	Reference
T5-1	—	DR3β1,DR52a,DQw2,DP4.1	DR1,DQw1,DP4.1	(13)
8.1.6	T5-1	DR3β1,DR52a,DQw2,DP4.1	— — DP4.1	(13)
9.22.3	8.1.6	— — DQw2,DP4.1	— — DP4.1	(10)
9.4.3	8.1.6	— DR52a,DQw2,DP4.1	— — DP4.1	(10, 17)
9.5.3	8.1.6	DR3β1,DR52a,DQw2,DP4.1	— — DP4.1	(10)
9.10.3	8.1.6	DR3β1,DR52a,DQw2,DP4.1	— — DP4.1	(10)
6.1.6	T5-1	— — — —	— — —	(12)

RESULTS AND DISCUSSION

We produced three alloreactive anti-DR3-specific clones by either repeated stimulation in vitro with a HLA-DR3 B lymphoblastoid cell line or by a mixed lymphocyte reaction. T cells were cloned by limiting dilution; a preliminary specificity analysis of the clones indicated they were specific for the DR3-DRw52a haplotype. Three clones (4.26A) were strongly reactive to several DR3 (w17) homozygous and heterozygous cell lines, and were not stimulated by the closely related DR5 haplotype or haplotypes, clone 4.26A also failed to respond to a split of DR3 (DR3w18) and all other known non-DR3 haplotypes (A. Johnson, unpublished observations). The fine specificity of the T cell clones was determined by their reactivity with DR3 mutants which have lost expression of one or both DRB genes. Four clones (24F7, 62D10, 4.26A, and C6) were specific for DR3β chains by virtue of their strong reactivity with 8.1.6, which expresses DR3, DQw2, and DPw4, and their failure to be stimulated by mutant 9.22.3, which lacks DR molecules but retains all other class II and class I MHC products (14). DR3 haplotypes code for a single DRα chain which forms dimers with each of two DRβ chains, DRβ1 and β3. The specificity of three of the clones (24F7, 62D10, 4.26A) could be further defined as against DR3β1; mutant 9.4.3, which has lost DR3β1 chain mRNA but retains normal levels of β3 molecules (21), fails to stimulate these three alloproliferative T cell clones. The fourth clone, C6, has been previously characterized and is specific for a DR52β chain determinant. It is stimulated by mutant 9.4.3, but not the DR null mutant, 9.22.3. (Fig. 1) (19). The four T cell clones were derived from three separate stimulation protocols and three different donors, and thus should be representative of anti-DR3 allospecific T cell clones.

Having defined the specificities of these anti-DR3 alloreactive clones, we asked whether they could be stimulated by the DR3 positive, Ag-presentation defective mutants which have lost the ability to form MHC class II peptide complexes. Both of the Ag presentation mutants tested (9.5.3 and 9.10.3) were unable to stimulate the three T cell clones specific for DR3β1 (Fig. 1). The fourth clone, C6, is stimulated by both of the presentation-defective APC. Clone C6 may recognize the foreign MHC molecule directly or may recognize a peptide-MHC complex which is generated by a pathway distinct from that affected in either of the two processing defective APC.

The inability of the presentation defective APC to stimulate the three DR3β1 allospecific clones appeared to be an absolute, rather than merely a quantitative effect. The response of the T cell clones to the progenitor APC 8.1.6 was strongly dependent on the number of stimulator cells. In contrast, there was no response to the mutant APC at numbers 4- to 16-fold above that required for a

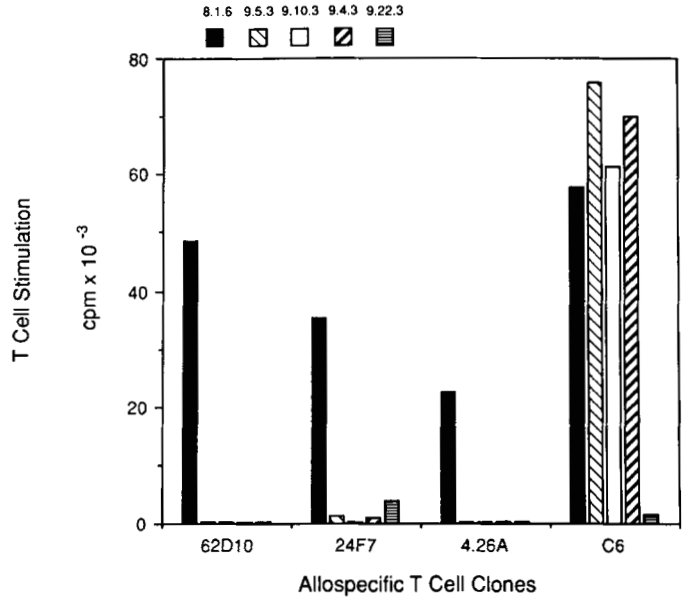

Figure 1. Stimulation of alloreactive T cell clones by Ag processing and presentation defective B cells. Four T cell clones specific for HLA-DR3 (62D10, 24F7, 4.26A, and C6) were stimulated with the DR3-expressing progenitor cell line, 8.1.6, and with four mutants derived from 8.1.6. Mutants 9.5.3 and 9.10.3 are defective in Ag processing and presentation of all intact protein Ag tested (11). Mutant 9.4.3 lacks the DR3β1 chain but expresses DR52a chain (17), and mutant 9.22.3 has a homozygous DRα deletion (10) resulting in neither DRβ chain being expressed at the surface.

detectable response (Fig. 2). We also investigated the possibility that the failure of the mutants to stimulate the DR3β1 allospecific clones might have been caused by loss of expression of the accessory molecules ICAM-1 or LFA-3. However, this does not appear to be the case; the levels of ICAM-1 and LFA-3 in progenitor 8.1.6 and mutants 9.5.3 and 9.10.3 are approximately equivalent (Fig. 3). Conformational changes in class II molecules that perturb the site of interaction of CD4 with class II molecules, or the interaction of class II molecules with T cell receptors in general, might result in loss of alloreactivity. However, it is unlikely that an effect on these interactions accounts for the inability of the mutants to stimulate alloreactive T cells for two reasons. One, the mutants can present antigenic peptides, as contrasted with intact exogenous Ag, to any of several different Ag-specific T cell clones (15). Two, they can stimulate some allospecific clones, such as C6 (Fig. 1).

Our results have established a link between the ability of stimulator cells to generate class II-peptide complexes and their ability to be recognized by at least some class II-restricted alloreactive T cells. Very little is known about the possible involvement of Ag processing in MHC class II-restricted allorecognition. Alloreactive T cell responses have been reported to be insensitive to the effects of

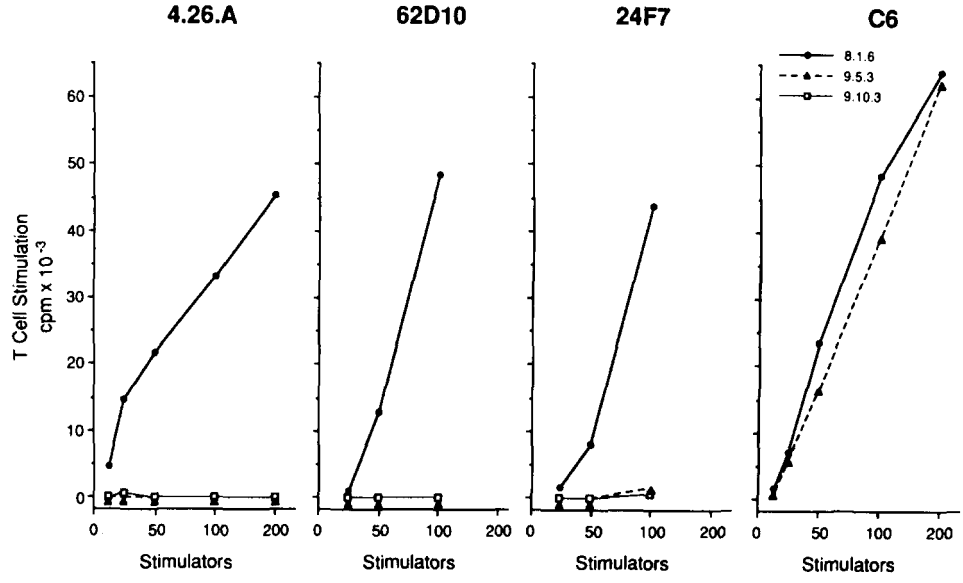

Figure 2. Dose-response of stimulation of alloreactive T cell clones by progenitor and mutant B cells. Three T cell clones specific for DR3β1 molecules were stimulated with various numbers of mitomycin C-treated B cell lines as described in Figure 1. 8.1.6 is the progenitor cell line of mutants 9.5.3 and 9.10.3, which are defective in Ag processing and presentation. Clone 4.26A had stimulation indices of >5.0 at all doses of stimulator cells; the response of C6 to 12,500 stimulators and the responses of both 24F7 and 62D10 to 25,000 stimulators were not statistically different than control responses. All four T cell clones exhibited a high threshhold requirement for the number of stimulator cells, a characteristic often noted for alloreactive T cells (5).

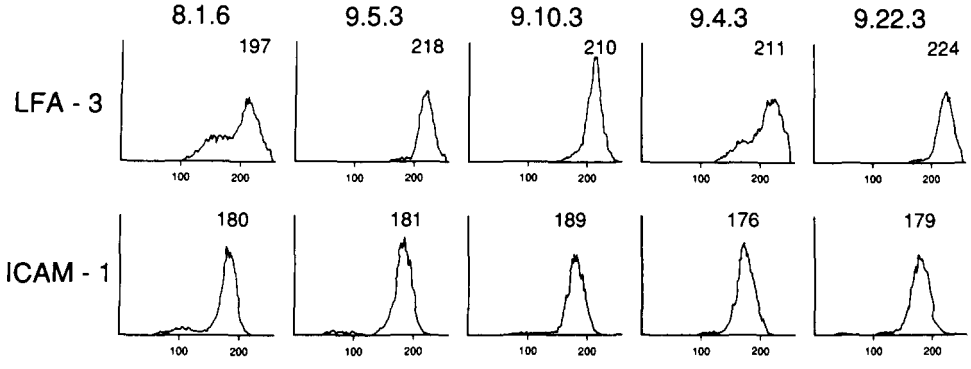

Figure 3. Expression of LFA-3 and ICAM-1 on parental B cell line 8.1.6 and its derivatives. The biphasic distribution of LFA-3 is occasionally seen (cf. 8.1.6). The modes of the major peak for 8.1.6, 9.5.3, and 9.10.3 were 213, 220, and 215, respectively. The mean of control staining of the rat thymoma C58 by mAb 60.3 was 95, and the mean 8.1.6 staining with the FITC goat anti-mouse IgG alone was 107.

chloroquine at doses that inhibited the processing and presentation of conventional Ag (24); however, because the exposure to chloroquine in that study was brief, these data may not be an adequate test of the requirement for Ag processing in allorecognition. Alternatively, the alloreactive T cells used in that study, like clone C6 here, might have been insensitive to disruptions of the exogenous Ag processing pathway. In this context, it is significant that MHC class II molecules are capable of presenting some endogenous protein Ag via a pathway that is insensitive to chloroquine (25, 26). Thus the failure to observe requirements for some alloreactive T cells may be that such alloreactive T cells are specific for peptides derived from Ag processed in the endogenous pathway. We have recently found that four additional alloreactive T cells, specific for DQ or DP, also fail to recognize the processing/presentation mutants; these findings support the possibility that many alloreactive T cells are sensitive to the occupancy of the peptide-binding groove (T. Cotner, unpublished observations).

The failure of these B cell mutants to stimulate three of four alloreactive T cell clones suggests that polymorphic residues in the class II molecules per se are not sufficient to stimulate all alloreactive T cells. Alloreactive T cell clones could recognize either MHC class II-peptide complexes or, because the MHC class II molecules in the mutant cell lines exhibit subtle conformational alterations (15), could recognize a conformation-sensitive MHC determinant. We have previously reported data which support the notion that the "normal" conformation of class II molecules may require that the peptide-binding site be occupied. This idea is based on the fact that the conformation of class II molecules is altered in each of the nine independent mutants which are defective in the ability to form MHC-peptide complexes. The class II molecules from these mutants can bind and present peptides provided extracellularly (15), making it unlikely that an altered conformation of class II molecules is the primary defect which prevents them from binding intracellularly generated peptides. The conformational change thus appears to be secondary to the inability of the mutants to form MHC-peptide complexes. Thus, we propose that peptide could be involved either directly in allorecognition, or indirectly by nature of the effect of peptide-binding on the conformation of class II molecules. Townsend et al. (25) have reported evidence suggesting that peptide binding may influence the expression of a stable MHC class I molecule. It is thus possible that peptide binding is a general requirement for the folding and stabilization of MHC molecules.

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