Corecognition of HLA-A1 and HLA-DPw3 by a Human CD4+ Alloreactive T Lymphocyte Clone

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Summary
We have generated an alloreactive proliferative T cell clone that only is stimulated by HLA-DPw3+ antigen presenting cells (APC) that at the same time carry HLA-A1. The T cell clone is CD4+, and the proliferation is blocked by anti-DP monoclonal antibodies and not by antibodies towards other class II or towards class I molecules. Family studies show that APC with A1 and DP3 on different haplotypes (trans) are able to stimulate the clone, and an HLA recombinant family gives evidence that the class I-carrying part of the haplotype is necessary for stimulation to occur. Stimulation is also observed with mixtures of APC expressing DPw3 and APC expressing A1, and likewise, DPw3+ APC become stimulatory when preincubated with supernatants from A1-positive cells. Our studies suggest that major histocompatibility complex (MHC) class I peptides presented by class II are allostimulatory and that APC can process MHC molecules that presumably are presented as allele-specific peptides in the context of other MHC molecules. We hypothesize that presentation of MHC peptides by MHC molecules constitutes an important part of alloreactive phenomena in vivo and in vitro.

Materials and Methods

Generation of T Cell Clones. PBMC from a responder "B" with type HLA-A2,25; B7,44; DR1,4; DQw1,3; DPw4 were cocultured with an equal amount of irradiated (20 Gray = 2,000 rad) PBMC from a donor "DL" HLA-A1,28; B7,8; DR1,4; DQw1,3; DPw3 (at 10^6 cells/ml) in culture medium (RPMI 1640 supplemented with 2 mM L-glutamine, 80 ng/ml streptomycin, 200 IU/ml penicillin, and 10% inactivated pooled human serum). After 6 d of culture in a humidified atmosphere at 37°C in 5% CO2, the blasts were purified over a 35-45% Percoll gradient and cloned in round-bottomed microtiter plates (Nunc, Roskilde, Denmark) at 0.5 cells/well with 10^6 irradiated (100 Gray) lymphoblastoid cell line (LCL) cells from the original stimulator in a total volume of 150 μl of culture medium. 3 d later, 10% of T cell growth factor-rich supernatant (TCGF sup) was added. At day 12, the positive wells were transferred to 2-ml well plates and restimulated with 10^6 irradiated (100 Gray) lymphoblastoid cell line (LCL) cells from the original stimulator in a total volume of 150 μl of culture medium. 3 d later, 10% of T cell growth factor-rich supernatant (TCGF sup) was added. At day 12, the positive wells were transferred to 2-ml well plates and restimulated with 10^6 irradiated LCL from donor DL. The culture medium containing 10% TCGF sup was changed three times a week, keeping the cell concentration <5 x 10^5 cells/ml. Every 12 d, the clones were restimulated until sufficient amounts were obtained, whereupon the cloned cells were cryopreserved in liquid nitrogen at the end of restimulation cycles.

Proliferative Assays. The thawed clones were plated at 10^4 cells/well in round-bottomed microtiter plates in the presence of 2.5 x 10^6 irradiated (100 Gray) LCL cells or 5 x 10^5 irradiated (20 Gray) PBMC in a total of 150 μl of culture medium. After 48 h, the cultures were labeled with 1 μCi/well of [3H]thymidine; 16 h later, the cells were harvested on glass filter by a semiautomatic harvester (Skatron, Lierbyen, Norway), and the incorporation of labeled thymidine was quantitated by liquid scintillation spectrometry. All cultures were done in triplicates and the median counts per minute was used for evaluations.

Mixtures of APC. The irradiated (20 Gray) PBMC or (100 Gray) LCL from two stimulators were mixed in various proportions,
mixed cells (2.5 \times 10^4 of LCL and 5 \times 10^4 of PBMC) were incubated at various times at 37°C in 5% CO₂ in round-bottomed microtiter plates in 100 µl culture medium before addition of 10⁴ clone cells in 50 µl of culture medium. The proliferative assay was then carried as described above.

*mAb Inhibition Assays.* Four mAbs were used: L243 that reacts with monomorphic determinants on DR molecules; TU22 (anti-DQ); B7/21 (anti-DP); and W6/32 (anti-HLA-class I). The blocking assay was carried out according to the 10th workshop protocol with at least four different fivefold dilutions (6).

**Results and Discussion**

Of 20 proliferative clones obtained from the priming (see above), six could be expanded to reasonable number in order to allow extended testings. The clone B/DL3, which is CD4⁺, showed a peculiar restimulation pattern. In a panel (7) of lymphoblastoid cell lines (LCL) from the 10th workshop, it appeared that all stimulatory APC carried HLA-A1 in addition to the HLA-DPw3 antigen. This reactivity pattern was confirmed in a local panel, and >60 different stimulator cells have been tested repeatedly with similar results. The reactivity of a subclone obtained by limiting dilution was identical when tested on a small selected panel.

Testing in families showed that when A1 and DPw3 were present on different haplotypes, only cells from individuals that were at the same time A1 and DPw3 stimulated the clone. In the family shown in Fig. 1, one individual had a recombination between HLA-A and HLA-DR, and this provided evidence that the HLA class I carrying part of the haplotype was needed in order to give stimulation. The A3 and A11 antigens that are serologically crossreactive with A1 did not give any stimulation when present on DPw3⁺ cells.

Blocking experiments with mAbs showed that only anti-DP antibodies were able to block the stimulation; anti-DR, anti-DQ, or anti-class I antibodies had little effect (Fig. 2).

Mixing experiments were performed with APC carrying different combinations of class I antigens and DP antigens. Mixtures of cells being DPw3 and cells being A1 were indeed able to stimulate the clone and this in a dose-dependent way (Fig. 3). Preliminary experiments showed that a 4-h preincubation of the stimulating cells was optimal and that LCL as well as PBMC could be used as APC in this and the other experiments mentioned. A strong stimulation of the clone was obtained when DPw3⁺ APC were preincubated with supernatant from an A1⁺ LCL and not with supernatant from an A1⁻ LCL (Table 1). This supernatant apparently contains a sufficient amount of A1 molecules, which after processing by the DPw3⁺ APC, are presented as peptides on DPw3 molecules. We have tested two peptides specific for A1 (amino acids 65–84 and 143–158), but these peptides do not stimulate our clone when preincubated with DPw3⁺ APC, and other peptides are under construction.

Several recent publications demonstrate that HLA class I peptides can be recognized when presented on class I molecules of another specificity (3) or that such peptides may inhibit alloreaction (4, 5). Likewise, viral peptides are able to inhibit alloreaction (8). Our findings suggest that the processing of class I peptides is a normally occurring in vitro phenomenon in APC and that class II molecules are able to present class I peptides.

Two earlier reports deal with CD4⁺ T cell clones having a specificity correlating closely with a class I specificity, al-

| Workshop no. | B/DL3 | Control reagent |
|--------------|-------|-----------------|
| STEINLIN (A1,DPw3) | 9057 | 10,982 8,634 11,445 38,325 39,895 31,430 |
| SLE005 (DPw3) | 9059 | 24,871 124 97 45,150 39,827 42,710 |
| LOO81785 (DPw3) | 9018 | 12,209 444 555 42,338 40,730 43,636 |
| BM21 (A1) | 9043 | 0 0 0 45,597 41,339 45,247 |
| BOLETH | 9031 | 0 0 0 22,795 20,982 24,954 |

The test shows that the clone is stimulated by DPw3⁺ APC when these are preincubated with supernatant from A1⁺ LCL (A1⁺ sup). Figures are median counts per minute of triplicate cultures, experimental value minus the value of stimulator cells alone. A zero indicates that the experimental value is equal to or lower than that obtained with stimulator cells alone. The culture supernatants were from two LCLs, positive (A1⁺ sup) and negative (A1⁻ sup), respectively, for A1. The supernatant was collected after 3 d of culture at a concentration of ~10⁴ cells/ml, filtered through a 0.22-µm filter, and 50 µl was added to the stimulator cells. After an incubation at 37°C for 4 h, the clone or control reagent was added and the proliferative test carried out as previously described. The HLA type of the stimulator cells: STEINLIN: A1,B8,DR3,Dw3,DQw2,DPw3/4; LOO81785: A3, B18,DR3,Dw3,DQw2,DPw3; SLE005: A2,B60,DRw13,Dw19, DQw6,DPw3; BM21: A1,B41,DRw11,Dw5,DQw7,DPw2; BOLETH: A2,B62,DR4,Dw4,DQw8,DPw4. The control reagent is a polyclonal T cell line raised against a pool of stimulators.
The proliferative responses of the clone B/DL3 against the LCL from an informative family. The figures are median counts per minute of triplicate cultures. Haplotypes in the family: a: HLA-A2, B17, DRw6, DQw1, DPw3; b: HLA-A1, B17, DR7, DQw3, DPw3; c: HLA-A1, B8, DR3, DQw2, DPw2; d: HLA-A9, B40, DRw6, DQw1, DPw2; a'b: HLA-A2, B17, DR7, DQw3, DPw3. A recombination between the A and DR locus on the chromosome inherited from the father was observed in the child no. 6. The cells from this child fail to stimulate the clone B/DL3. The presence of HLA-A1 and DPw3 at the same time coincides with a strong stimulation, this combination can be in cis (nos. 1 and 7) or trans (nos. 3, 4, and 5). A weak stimulation by the cells of the mother is probably due to the presence of A1 (see text).

T cell clones often display a reaction pattern that correlates closely with a specificity defined by other methods (6), and in such cases, the peptide presented by MHC is probably a nonpolymorphic peptide originating from an autologous protein that may be tissue specific (13). Other clones have reactions that are difficult to reconcile with a certain specificity or epitope, even with knowledge of amino acid sequences of the MHC molecule in question. Such reactions usually segregate with HLA haplotypes in families but may also appear to depend on two different HLA haplotypes (14). It is tempting to speculate that such atypical results are due to the presentation of a polymorphic MHC peptide, either class I or II, by another MHC molecule.

Our hypothesis also explains some curious results on T cell clones apparently stimulated by hybrid antigens (15, 16). One of these reports (15) deals with a clone that only was stimulated by cells that were at the same time DR2 and DR4. As the Met chain is nonpolymorphic, it was assumed that the stimulatory molecule was a heterodimer consisting of a DRα chain and a DQβ chain. Although the existence of such molecules has been suggested in other cases by biochemical studies (17), the blocking studies performed by mAbs were incompatible with such hybrid heterodimers. A more plausible explanation is the presentation of a polymorphic DQ (or DR) peptide by a DR molecule.

A recent report (18) describes T cell clones reactive with a DR3 peptide presented by DPw3. This indicates that the same class II molecule (DPw3) has the possibility to bind different HLA peptides. Examples of presentation of class II
peptides by class I molecules have not yet been reported, but it can be speculated, as recently suggested by Janeway (19), that this might happen in T cells and constitute the molecular basis for T cell suppressor networks.

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