gene can now be compared with those of men with multiple long-wave genes. The absolute difference from the mean (mean = 0.50) was determined for each R/(R + G) value given in Table 1. The absolute deviation from the mean color match was, on average, larger for the men with only one long-wave gene (0.056) than for those with multiple long-wave genes (0.032). This difference is unlikely to have arisen by chance (Mann-Whitney U test = 42.5; $P = 0.02$). The fact that individuals with multiple long-wave genes tend to make more intermediate color matches hints that some may be expressing more than one long-wave pigment. If that is true, in total they would express at least four different cone pigments: two long-wave, a middle-wave, and a short-wave pigment. This would seem counter to the Young-Helmholtz theory that has held sway for more than two centuries, in which the presence of three pigments is proposed to explain human trichromatic color vision. In any case, we can now seek to understand how normal color vision phenotypes can arise from such an enormous variety of genotypes and to reexamine how the normal genotypes might have been altered to result in color vision defects.

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Peptide Specificity in the Recognition of MHC Class I by Natural Killer Cell Clones

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Recognition by natural killer (NK) cells of major histocompatibility complex (MHC) class I molecules on target cells inhibits NK-mediated lysis. Here, inhibition of NK clones by HLA-B*2705 molecules mutated at single amino acids in the peptide binding site varied among HLA-B*2705–specific NK clones. In addition, a subset of such NK clones was inhibited by only one of several self peptides loaded onto HLA-B*2705 molecules expressed in peptide transporter–deficient cells, showing that recognition was peptide-specific. These data demonstrate that specific self peptides, complexed with MHC class I, provide protection from NK-mediated lysis.

Natural killer cells are a distinct lymphocyte population that controls the early phase of infection by various pathogens such as viruses, intracellular bacteria, protozoa, and fungi (*1*). Natural killer cells can eliminate infected cells by direct cell lysis, but they also influence subsequent T cell responses by secreting potent mediators of inflammation (*1*). Class I molecules of the MHC expressed on target cells play an important role in the specificity of recognition by NK cells (*2–5*). Engagement of NK receptors for class I molecules turns off the lytic machinery and lymphokine production (*3, 5–7*). In humans, several NK molecules are involved in the recognition of human leukocyte antigen (HLA)–C (*6*) and HLA-B (*8*) alleles. However, the nature of the specific interaction between NK receptors and target class I molecules remains obscure. For example, it is still not known whether NK cells recognize a conventional trimolecular class I complex of heavy chain, β_2-microglobulin, and peptide or other, less stable conformations of class I (*9*). Here, the well-defined HLA-B*2705 allele was used as a tool to test how NK cells recognize class I molecules.

To isolate NK clones specific for HLA-B*2705, we generated a large panel of clones (*10*) from two individuals and screened the clones for their ability to lyse the B cell line C1R, which expresses HLA-Cw4 as the only serologically detectable allele (*11*), and its transfected derivative C1R-B*2705 (*12*). As expected from previous work (*5, 13*), B*2705 molecules on C1R cells provided protection from NK lysis by several, but not all, NK clones. The specificity of 10 randomly chosen B*2705-specific NK clones was assessed further with two other C1R transfectants (*14*) that express equivalent surface amounts of B*0801 or B*1402 molecules (Fig. 1A). Seven clones (including 2wA-08 and 2wA-29) recognized only B*2705, whereas three clones (such as 10wA-16) also recognized B*0801. Protection by several HLA-B alleles has been reported with other NK clones (*5*), perhaps the result of expression of several receptors on a single NK clone, or cross-reactivity of NK receptors with related class I alleles.

To show that B*2705 molecules were directly involved in recognition by the NK clones, we used monoclonal antibody (mAb) A6/136 (anti–class I) (*15*) to interfere with class I–mediated protection. At saturating conditions, A6/136 inhibited the protection from lysis—that is, it restored lysis—by all six NK clones tested (three are shown in Fig. 1B). A control isotype-matched immunoglobulin M (IgM) mAb had no effect. The IgM mAb B27.2M (anti–HLA-B27) (*15*) restored lysis by only one out of the six clones tested (2wA-08 in Fig. 1B), suggesting that NK clones can recognize B*2705 molecules in different ways.

Functional heterogeneity among B*2705-specific NK clones was shown by use of B*2705 molecules mutated at single amino acid residues in the peptide binding site (Fig. 2). Surface expression of mutants E45T, L95I, and D116F (*16*) transfected in C1R cells was comparable to that of wild-type B*2705 molecules, except for mutant E45T, whose expression was lower (Fig.

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2A). Recognition of these mutants varied greatly among NK clones (Fig. 2B). For instance, two clones (2wA-08 and 10wA-17) shared the same recognition pattern, that is, protection maintained by mutant L95I but not by the other two mutants. With clone 10wA-16, in contrast, protection was maintained with mutant E45T, in spite of the low surface expression, but was lost partially with mutant L95I and completely with D116F. Residues E45 and D116 contribute substantially to the stabilization of bound peptides (*17*). In vitro assembly (*18*) and in vivo binding (*19*) assays revealed differences in both the stability and type of peptides bound to the mutated molecules E45T and D116F. These data and our findings suggest that peptides bound to class I molecules contribute to the specificity of target cell recognition by NK cells.

To demonstrate directly the role of peptides in providing protection from NK lysis, we loaded class I molecules on cells deficient in the transporter for antigen presentation (TAP) with defined peptides. This approach was made possible by the identification of self peptides associated with B*2705 molecules (*17*) and by the isolation of specific NK clones. Previous studies with NK populations reported a partially increased sensitivity of target cells incubated with exogenous peptides (*20*). Such data do not imply a direct role of peptides in protection from NK cells, because binding of exogenous peptides to class I may have unmasked a NK-triggering target structure (*2*). We used the TAP2-deficient mouse cell line RMA-S cotransfected with the human β_2-microglobulin and B*2705 genes (*21*), and the human cell line T2, which lacks the entire class II MHC region, transfected with B*2705 (*21*) (Fig. 3A). Peptide loading at the cell surface was maximized by a combination of low temperature (*22*) and high doses of peptide. Incubation of RMA-S–B27 cells at 26°C alone led to a doubling of surface B*2705 molecules. Further addition of any one of four synthetic peptides during the 26°C incubation resulted in a five- to sixfold increase of surface B*2705 molecules (Fig. 3A). Three of the peptides correspond to predominant peptides eluted from B*2705 molecules in a wild-type B cell line (*17*). The fourth was a synthetic peptide that promotes efficient in vitro assembly of B27 molecules (*18*). Even though turnover of these reconstituted B*2705 molecules was rapid upon return at 37°C, amounts substantially higher than initial levels at 37°C remained after 3 hours (Fig. 3A), which corresponds to the length of the cytotoxicity assay.

The ability of B*2705 molecules that were loaded with specific peptides to provide protection from NK lysis was tested with four NK clones (Fig. 3B). As expected for TAP-deficient cells (*23*), RMA-S–B27 cells were lysed by the NK clones. In contrast, RMA-S–B27 cells incubated with one of the four peptides became protected from two of the four clones. These two NK clones were also related to each other by their sensitivity to mutations in the peptide binding site (Fig. 2). Two other clones did not recognize B*2705 molecules loaded with peptide 1, and none of the clones recognized B*2705 molecules loaded with the other three peptides (Fig. 3B). The unique ability of peptide 1 to restore a protective conformation to

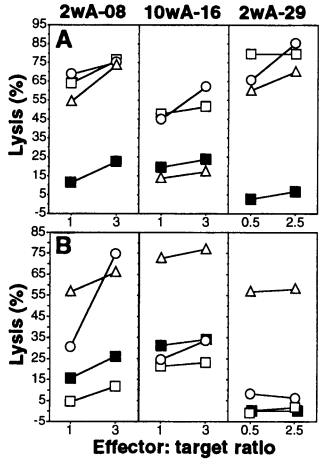

Fig. 1. Specific recognition of HLA-B*2705 by human NK clones. (**A**) Lysis of untransfected C1R cells (○) and of C1R cells transfected with HLA alleles B*2705 (■), B*0801 (△), or B*1402 (□) by the three indicated NK clones (*10*). (**B**) The protection conferred by B*2705 is abrogated by some anti–class I antibodies (*15*). Lysis of C1R-B*2705 cells was assayed in the absence of Ab (■) or in the presence of a saturating amount of mAb A6/136 (△), mAb B27.2M (100 μg/ml) (○), or an isotype-matched irrelevant IgM (100 μg/ml) (□).

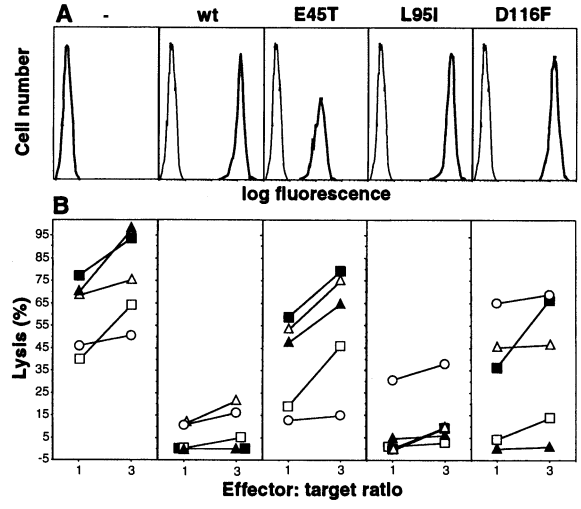

Fig. 2. NK clones are differentially sensitive to single amino acid substitutions in the peptide-binding site of the B*2705 molecule. (**A**) Surface expression of B*2705 molecules on C1R cells (−) or C1R cells transfected with wild-type B*2705 (wt) or with mutants E45T, L95I, and D116F, as indicated. These mutants have been described (*19*). Cells were incubated with the B27-specific mAb ME.1 (*15*), washed, and incubated with a goat antibody to mouse IgG1 conjugated with phycoerythrin and analyzed by flow cytofluorimetry (FACScan, Becton Dickinson). Profiles on the left represent cells incubated with the second reagent alone. (**B**) Lysis of the indicated cells by NK clones 2wA-08 (△), 10wA-16 (○), 2wA-29 (▲), 2w-14/C1R (□), and 10wA-17 (■). Similar data were obtained in two other independent experiments.

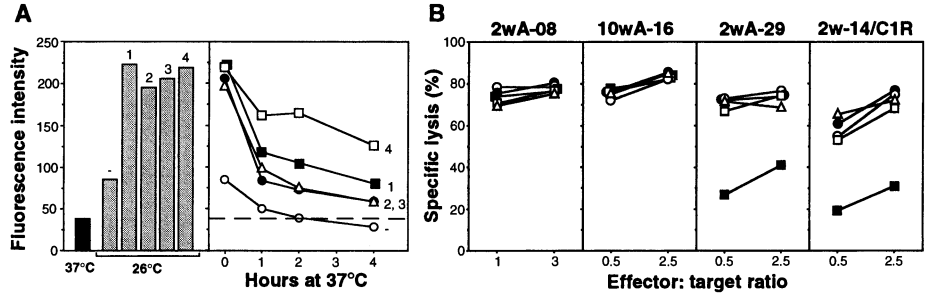

Fig. 3. Peptide-specific recognition of B*2705 by NK clones. (**A**) Peptide-induced stabilization of B*2705 molecules on RMA-S–B27 cells incubated at 37°C (filled bar) or at 26°C for 24 hours (shaded bars) without (−) or with peptide 1, peptide 2, peptide 3, or peptide 4, as indicated. These peptides have been described (*26*). Peptide (100 μM) was added at the onset of the culture and another 100 μM added after 12 hours. Surface B27 expression was determined as in Fig. 2. Cells without peptide (○) or cells incubated with peptide 1 (■), peptide 2 (△), peptide 3 (●), or peptide 4 (□) were washed, incubated at 37°C for the indicated time, and assayed for surface B27. The dashed line represents B27 expression at 37°C. (**B**) Lysis by the indicated NK clones of RMA-S–B27 cells incubated with peptides as described in (A).

B*2705 was not due to a quantitative effect, because peptide 4 generated a more stable conformation of B*2705 molecules (Fig. 3A) yet did not restore protection from any NK clone (Fig. 3B). The protective effect of peptide 1 required B*2705 expression: Untransfected RMA-S and T2 cells incubated with peptide 1 were still lysed by NK cells (Fig. 4). Data obtained with T2-B27 cells confirmed the protective effect of peptide 1 with clones 2wA-29 and 2w-14/C1R (Fig. 4).

The requirement for a specific peptide in the formation of a protective structure virtually eliminates the possibility that NK cells are triggered by a target structure bound to class I and released by peptide binding, as postulated by the masking hypothesis (*2*). The peptide specificity in class I recognition by NK cells can explain the heterogeneity among NK clones in their ability to lyse virus-infected targets (*24*): The loss of protection from NK lysis caused by virus infection no longer implies replacement of most endogenous peptides but could occur through interference with the formation of specific class I–peptide complexes.

Our data show that a fully assembled class I molecule is competent for an effective interaction with NK receptors, as measured by the protection of target cells from NK-mediated lysis, and that peptides contribute directly to such protective conformations. In addition, NK cells exhibit peptide specificity, as suggested by the distinct sensitivity of different NK clones to single amino acid changes in the peptide binding site and as demonstrated by the ability of two NK clones to discriminate among four peptide-dependent configurations of the same B*2705 molecule. These results also imply the existence of multiple NK receptors able to distinguish among different class I–peptide complexes.

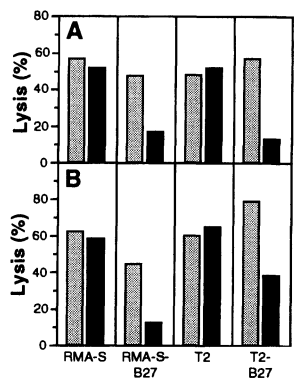

Fig. 4. The protection from lysis provided by peptide 1 is dependent on B*2705 expression, both in RMA-S and in T2 cells. (**A**) Lysis by NK clone 2wA-29 (effector:target ratio of 0.5) of the indicated cells incubated without (shaded bars) or with peptide 1 (filled bars) as in Fig. 3. (**B**) Lysis by NK clone 2w-14/C1R as in (A).

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15. The IgM mAb A6/136 was isolated on the basis of its ability to abrogate the class I–mediated protection of target from lysis by NK cells (*25*). The IgM B27-specific mAb B27.2M and the IgG1 B27–specific mAb ME.1 were obtained from American Type Culture Collection, Rockville, MD.
16. E45T indicates the replacement of the glutamic acid residue 45 by threonine. L, leucine; I, isoleucine; D, aspartic acid; and F, phenylalanine. A more extensive study with a larger number of NK clones and additional mutants of B*2705 will be reported elsewhere.
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Correlation of Terminal Cell Cycle Arrest of Skeletal Muscle with Induction of p21 by MyoD

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Skeletal muscle differentiation entails the coordination of muscle-specific gene expression and terminal withdrawal from the cell cycle. This cell cycle arrest in the G_0 phase requires the retinoblastoma tumor suppressor protein (Rb). The function of Rb is negatively regulated by cyclin-dependent kinases (Cdks), which are controlled by Cdk inhibitors. Expression of MyoD, a skeletal muscle–specific transcriptional regulator, activated the expression of the Cdk inhibitor p21 during differentiation of murine myocytes and in nonmyogenic cells. MyoD-mediated induction of p21 did not require the tumor suppressor protein p53 and correlated with cell cycle withdrawal. Thus, MyoD may induce terminal cell cycle arrest during skeletal muscle differentiation by increasing the expression of p21.

Myogenic basic helix-loop-helix (bHLH) proteins, such as MyoD, promote skeletal muscle–specific gene expression and permanent cell cycle arrest (*1*). Forced expression of MyoD can inhibit cell cycle progression independently of muscle differentiation (*2*), but the molecular basis for this effect and its relation to differentiation of normal skeletal muscle are not known. Members of the retinoblastoma family of tumor suppressor proteins are important for myogenic differentiation. Their inactivation by viral oncoproteins both blocks differentiation and induces DNA synthesis in otherwise terminally differentiated myocytes [reviewed in (*3*)]. A specific role for Rb has been demonstrated as differentiated skeletal muscle cells express large amounts of hypophosphorylated (active) Rb (*4*, *5*), and specific loss of Rb through gene inactivation pre-