pulsed macrophages by using the macrophages pulsed with either gp96 preparation to stimulate CTLs against an irrelevant tumor None of the pulsed macrophages could stimulate the tumor specific CTLs

19 For in vitro reconstitution of gp96 peptide complexes gp96 derived from normal liver (50 μg) and ^{125}I labeled peptides (5 μg) were incubated at 50°C for 10 min followed by room temperature for 30 min Free peptides were removed by extensive washing with Microcon 50 (Amicon), such that no free peptides were detected on SDS–polyacrylamide gel electrophoresis of the complexes (Z Li R Suto P K Srivastava, in preparation)

20 The sequence of VSV20 is Ser Leu Ser Asp Leu Arg Gly Tyr Val Tyr Gln Gly-Leu Lys Ser Gly Asn Val Ser Cys The sequence of the negative control VSV peptide A is Lys Arg Gln Ile Tyr Thr Asp Leu Glu Met Asn Arg Leu Gly Lys

21 C57BL/6 mice (*H 2^b* haplotype) were subcutaneously injected twice at a 7 day interval with gp96 (10 μg in phosphate buffered saline) derived from uninfected or VSV infected Meth A cells or EL4 cells Seven days after the second vaccination spleens were removed and spleen cells (8 × 10^6 cells per well) were cocultured in mixed lymphocyte tumor culture (MLTC) with irradiated N1 cells (1 4 × 10^5 cells per well) in 24 well plates On day 7 each well was harvested Serially diluted culture cells were tested against N1 cells or EL4 cells for cytotoxicity in a ^{51}Cr release assay

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Human H-Y: A Male-Specific Histocompatibility Antigen Derived from the SMCY Protein

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H-Y is a transplantation antigen that can lead to rejection of male organ and bone marrow grafts by female recipients, even if the donor and recipient match at the major histocompatibility locus of humans, the HLA (human leukocyte antigen) locus. However, the origin and function of H-Y antigens has eluded researchers for 40 years. One human H-Y antigen presented by HLA-B7 was identified as an 11-residue peptide derived from SMCY, an evolutionarily conserved protein encoded on the Y chromosome. The protein from the homologous gene on the X chromosome, *SMCX*, differs by two amino acid residues in the same region. The identification of H-Y may aid in transplantation prognosis, prenatal diagnosis, and fertilization strategies.

Histocompatibility antigens that can induce transplant rejection include the class I and class II molecules of the major histocompatibility complex (MHC), as well as a large number of so-called minor histocompatibility (H) antigens In mice, the use of inbred strains has shown that minor H antigens are encoded by almost 50 different allelically polymorphic loci scattered throughout the genome (*1*) Humans also have minor H antigens although their overall number and complexity remains uncertain Both species have the male specific antigen H-Y (*2, 3*) H-Y was initially identified through the observation that within an inbred mouse strain, most of the male-to-female skin grafts were rejected, whereas transplants in other sex combinations nearly always succeeded (*2*) In humans, sex mismatch is a significant risk factor associated with rejection or the development of graft-versus-host disease in bone marrow transplant recipients (*3–6*) The H-Y antigen is expressed in most different human tissues (*4, 7*), and H-Y specific immune responses occur during the transplantation of other organs, blood transfusion, and pregnancy (*8*)

As with other minor H antigens, the recognition of H-Y by T lymphocytes is MHC-restricted (*3, 9*), and some H-Y antigens are peptides derived from cellular proteins that are presented on the cell surface in association with MHC class I molecules (*10*) We have developed a technique for the identification of individual peptides that are bound to MHC molecules and recognized as antigens by T cells By combining microcapillary liquid chromatography–electrospray ionization mass spectrometry with T cell epitope reconstitution assays (*11–13*) we now report the identification of a peptide antigen recognized by a human cytotoxic T lymphocyte (CTL) clone that is H-Y–specific and restricted by the class I MHC molecule HLA-B7

To isolate endogenously processed H-Y peptides, HLA-B7 molecules were purified by affinity chromatography from the H-Y positive, B lymphoblastoid cell line JY (*14*) The associated peptides were extracted in acid and separated from high molecular weight material by ultrafiltration (*15*) and subsequently fractionated by reverse-phase high-pressure liquid chromatography (HPLC) (*11*) Samples of each fraction were incubated with HLA-B7$^+$, H-Y$^-$ T2-B7 target cells to assay for reconstitution of the epitope recognized by

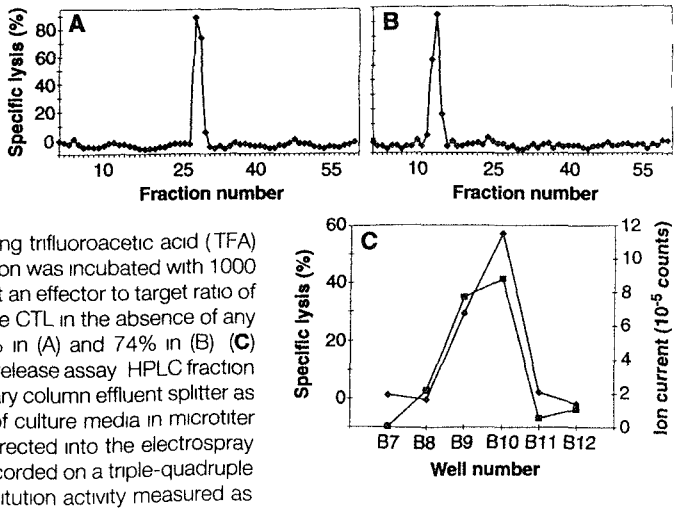

Fig. 1. Reconstitution of the H-Y epitope with HPLC-fractionated peptides extracted from HLA-B7 molecules (**A**) HLA-B7-molecules were immunoaffinity purified form 2 × 10^{10} H Y$^+$ JY cells Peptides were eluted from B7 molecules with 10% acetic acid, pH 2 1, filtered through a 5-kD cut-off filter and fractionated on a C18 reverse phase column Buffer A was 0 1% heptafluorobutyric acid (HFBA) and buffer B was 0 1% HFBA in acetonitrile The gradient consisted of 100% buffer A (0 to 20 min), 0 to 12% buffer B (20 to 25 min), and 12 to 50% buffer B (25 to 80 min) at a flow rate of 200 μl/min Sixty fractions of 200 μl each were collected from 20 to 80 min (**B**) Fractions 28 and 29 from the separation shown in (A) were rechromatographed with the same acetonitrile gradient, but using trifluoroacetic acid (TFA) instead of HFBA as the organic modifier For both panels, 3% of each peptide fraction was incubated with 1000 ^{51}Cr-labeled T2-B7 cells at room temperature for 2 hours CTLs were then added at an effector to target ratio of 10 to 1 and further incubated at 37°C for 4 hours Background lysis of T2-B7 by the CTL in the absence of any peptides was −3% in (A) and −4% in (B), positive control lysis of JY was 75% in (A) and 74% in (B) (**C**) Determination of candidate H-Y peptide by mass spectrometry combined with ^{51}Cr release assay HPLC fraction 14 from the separation in Fig 1B was chromatographed with an on-line microcapillary column effluent splitter as previously described (*11, 13*) One-fifth of the effluent was deposited into 100 μl of culture media in microtiter plate wells for analysis with CTLs The remaining four-fifths of the material were directed into the electrospray ionization source, and mass spectra of the peptides deposited in each well were recorded on a triple-quadruple mass spectrometer (Finnigan-MAT, San Jose, California) (◆) H Y epitope reconstitution activity measured as percent specific lysis (■), abundance of peptide 1171 measured as ion current at m/z 391

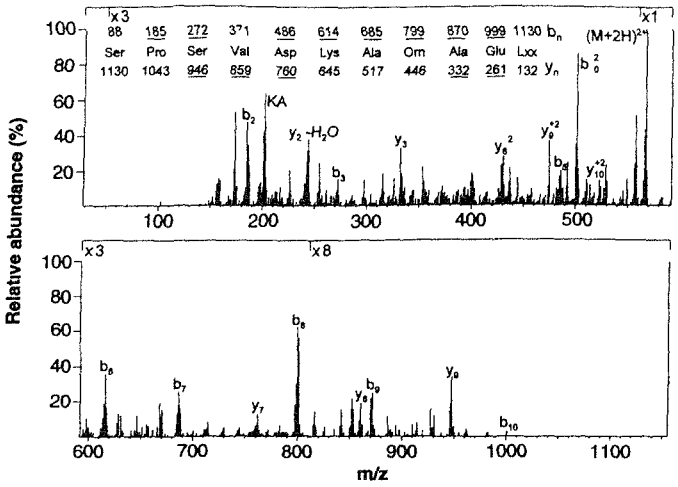

Fig. 2. The CAD mass spectrum of peptide 1171 after conversion the R residue to ornithine Material from second dimension HPLC fraction 14 shown in Fig 1B was treated with 70% hydrazine hydrate for 1 hour The CAD mass spectrum was recorded on the $M+2H)^{+2}$ ion at m/z 566

an HLA-B7 restricted, H-Y–specific CTL clone, 5W4 (*16*) A single peak of reconstituting activity was observed (Fig 1A), which was rechromatographed in a different organic modifier Although a single active peak of reconstituting activity was also observed from this separation (Fig 1B), it still contained more than 100 distinct peptide species, as assessed by electrospray ionization tandem mass spectrometry

To identify the active H-Y peptide in this mixture, we applied each active fraction separately to a microcapillary HPLC column and split the effluent after the separation (*11*) Four-fifths of the effluent was directed into the mass spectrometer for analysis, while one-fifth was simultaneously directed into a 96-well microtiter plate for a subsequent epitope reconstitution assay The amount of the H-Y sensitizing activity in each well was correlated to signals observed in the mass spectrum and therefore to the abundance of different peptide species By comparing the profile of H-Y activity and the ion abundance data (Fig 1C), we identified an $(M+3H)^{+3}$ ion at a mass-to-charge ratio (m/z) of 391 (neutral molecular mass=1171), whose abundance correlated with the amount of H-Y epitope reconstituting activity A peptide with an identical mass and collision-activated dissociation (CAD) spectrum was also present in HLA-B7–associated peptides extracted from a second $H\text{-}Y^{+}$ B lymphoblastoid line, DM, but absent from a spontaneous $H\text{-}Y^{-}$ loss variant of this cell, DM(−) (*17*)

Assignment of a complete amino acid sequence to peptide 1171 from the CAD mass spectrum recorded at the 20 fmol level proved difficult due to the absence of high mass fragment ions containing the amine terminus (b-type ions) A series of single, doubly charged, or both fragment ions that contained the COOH-terminus (y-type ions) identified the COOH-terminal residue as either L or I and the first six amino acids as SPSVDK (*18*) The difference in molecular mass between this partial sequence and that of the full length peptide suggested the presence of four additional residues, for a total length of 11 Because the candidate peptide existed exclusively in the gas phase as an $(M+3H)^{+3}$ ion, and underwent mass shifts of 42 and 84 Da on conversion to the corresponding methyl ester and acetylated derivative, respectively, two of the remaining residues were assigned as R and either D or E Only two combinations of four residues (AREA and GRDV) meet the above criteria and satisfy the missing mass of 427 Da The CAD spectra recorded on synthetic peptides suggested that R could not be located at either position 7 or 10 Data bases were searched for proteins containing peptides with these characteristics, and a sequence consistent at nine of 11 positions was found in residues 963 to 973 of the protein encoded by a gene called *XE169* or *SMCX* (*19*), which is located on the X chromosome A homolog of *SMCX*, called *SMCY*, is located on the Y chromosome (*20*) This protein (*21*) contains a sequence (residues 950 to 960) that is consistent at 11 out of 11 positions and has the expected mass of 1171 Da A CAD mass spectrum recorded on the naturally processed material after conversion of the R residue to ornithine confirmed that its sequence was identical to that found in the SMCY protein (Fig 2)

A synthetic peptide corresponding to the 11 residue SMCY sequence (SPSVDK-ARAEL) sensitized T2-B7 cells for recognition by the H Y specific CTL clone Half maximal lysis was achieved at a peptide concentration of 10 pM (Fig 3A) The corresponding peptide from the sequence of the X chromosomal homolog, SMCX, has substitutions of A for S at position 3 and Q for R at position 8 Although this peptide also was able to sensitize T2-B7 cells for recognition, comparable killing was only achieved with 10,000 times the peptide concentration The concentration of the SMCY peptide that inhibited the binding of an iodinated standard peptide to purified

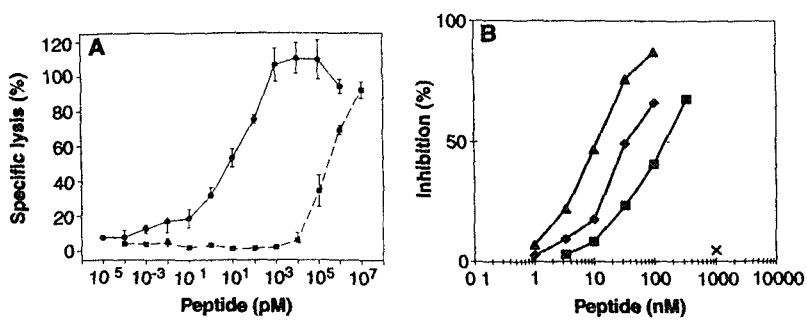

Fig. 3. Reconstitution of the H-Y epitope with synthetic peptides **(A)** Cytolytic assays ^{51}Cr release was assayed at an effector to target ratio of 10 to 1 on T2-B7 cells that had been incubated with the indicated concentration of SMCY peptide SPSVDKARAEL (◆), or SMCX peptide SPAVDKAQAEL (■) **(B)** Binding of synthetic peptides to purified HLA B7 HPLC-purified test peptides were assayed for the ability to inhibit the binding of the iodinated endogenous B7 peptides APRYTVLLL to purified HLA B7 as described (*28*) ◆, SMCY peptide SPSVDKARAEL ■, SMCX peptide SPAVDKAQAEL, ▲, APRTLVLLL an endogenous peptide bound to HLA-B7, and × LLDVPTAAV, an endogenous peptide bound to HLA A2 1 as the negative control Synthetic peptides were purified to homogeneity by reverse phase-HPLC on a Vydac C4 column Purity was established on an analytical RP column and the quantity of each peptide was confirmed by comparing the area of the peak with that of a standard peptide The identity of the peptides was confirmed by mass spectrometry

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HLA-B7 by 50% (IC_{50}) was 34 nM, whereas the IC_{50} for the SMCX peptide was 140 nM (Fig. 3B). Thus, the significant difference in the ability of the SMCY and SMCX peptides to sensitize targets for T cell recognition is almost entirely due to the fine specificity of the T cell receptor, rather than the differences in MHC binding affinities. The SMCX peptide was also present in naturally processed peptide extracts of HLA-B7, although its abundance was only 25% of the SMCY peptide abundance (*17*). Therefore the peptide epitope representing the HLA-B7–restricted H-Y antigen is derived from the protein encoded by *SMCY*

The location of the *SMCY* gene and the control of its expression fit well with those expected of the H-Y antigen based on previous work. Expression of SMCY has been detected in all male tissues tested, as has H-Y (*4, 7, 19*) Deletion mapping in humans has placed the *HY* locus in a portion of interval 6 on the long arm of the human Y chromosome (*22*), and *SMCY* maps to this same interval (*20*) Our work also establishes that the H-Y structural gene is encoded on the Y chromosome, rather than being an autosomal gene controlled by Y. The SMCY and SMCX proteins are 85% identical, and the *SMCX* gene is expressed from both the active and the inactive X chromosomes in both mice and humans (*19, 23*). Therefore, self-tolerance to SMCX will limit the number of SMCY peptides that could give rise to H-Y epitopes in association with different MHC molecules. On the other hand, SMCY contains almost 1500 residues, and the over 200 amino acid sequence differences between it and SMCX are scattered relatively uniformly throughout its length. Thus, a large number of distinct SMCY-specific peptides could be generated as H-Y epitopes. Whether the H-Y epitope peptides presented by other MHC molecules are also from SMCY is unknown, because genetic mapping of the mouse Y chromosome has suggested between two and five distinct loci encoding H-Y antigens (*24*). However, a murine H-Y epitope restricted by H-$2K^k$ has also been shown to be derived from the murine Smcy protein (*25*). The demonstration that two H-Y epitopes from either mouse or human are derived from the same protein makes SMCY the prime target in searching other H-Y epitopes.

The identification of the protein that gives rise to an H-Y antigen culminates 40 years of uncertainty regarding its origin and many attempts to identify it. The 77% DNA sequence identity between SMCY and SMCX provides a likely explanation for past failures to identify H-Y–encoding genes by subtractive hybridization. Both proteins share significant sequence homology to retinoblastoma binding protein 2, which has been suggested to be a transcription factor (*26*). If SMCY functions as such, its presumed intracellular location would be inconsistent with detection by male-specific antibodies that have been shown to recognize cell surface structures (*27*) Although the function of SMCY, as well as the homologous SMCX, remains unclear, this and other H-Y specific peptides are candidates for immunomodulatory approaches in bone marrow transplantation, genetic probes to be used for prenatal diagnosis in sex-linked congenital abnormalities, and investigating minimal residual disease and chimerism.

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18 Single-letter abbreviations for the amino acid residues are A, Ala, C, Cys, D Asp E Glu F Phe G Gly, H, His, I Ile K, Lys, L, Leu M, Met N Asn P Pro Q, Gln, R, Arg, S Ser, T Thr V Val W Trp and Y, Tyr
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TECHNICAL COMMENTS

Neutrophilia in Mice That Lack the Murine IL-8 Receptor Homolog

G. Cacalano *et al* describe neutrophil and B cell expansion in mice lacking the murine interleukin-8 receptor homolog (mIL-8Rh) (*1*). Neutrophils from these mice did not migrate toward ligands of the mIL-8Rh, and many fewer neutrophils arrived at sites of inflammation. These results could be expected, but the profound increase in the neutrophil and B cell populations was unexpected. Cacalano *et al* offer several possible explanations for this result, but strong evidence to support any one is lacking.

We would like to offer an alternative explanation, namely, that the neutrophil and B cell expansion are compensatory changes for poor resistance to normal flora and pathogen exposure. We base this argument on functional, histological, and clinical similarities between these mice and patients with leukocyte adhesion deficiency (LAD) Humans, dogs, and cattle can have LAD, and all afflicted individuals suffer a defect in the CD18 gene and lack expression of β_2-integrin adhesion molecules on their neutrophils (*2*). Consequently, neutrophils are unable to adhere to and cross the endothelium, so they cannot reach sites of infection Individuals with LAD may appear generally normal, especially when bacterial exposure is minimized, but they often suffer chronic, subclinical infections. Classical signs of LAD include gingival infection with abnormal dentition, and among cattle, growth retardation (*3, 4*). Increased size of lymphoid organs and profound persistent neutrophilia with extensive granulopoietic activity outside of the bone marrow are hallmarks of this disease (*5*). Similarly, persistent neutrophilia