

HLA EXPRESSION IN CANCER



Project Aspects

A. Specific Aims

Abnormalities in HLA Class I antigen expression in malignant cells are expected to play a role in the clinical course of the disease (for review, see 1) and to have a negative impact on T cell-based immunotherapy (2,3). There are conflicting data about the frequency of HLA Class I antigen abnormalities in malignant diseases and their clinical significance. Whether these discrepancies reflect lack of standardization of the immunohistochemical assays used and/or heterogeneity of the patient populations investigated is not known. To address these questions in a conclusive and timely fashion we have organized a study group within the 13th International Histocompatibility Working Group (IHWG). Utilizing a standardized methodology this group composed of the leading laboratories in the HLA and cancer field is expected to generate an amount of information which no individual laboratory can generate.

In the past, the large majority of the published studies have been performed by immunoperoxidase staining of frozen tissue sections with monoclonal antibodies (mAb) to monomorphic determinants of HLA Class I antigens (for review, see 1). Although these studies have yielded valuable information, testing of HLA Class I antigen expression in malignant lesions is not routinely used to evaluate patients, even those to be treated with T cell-based immunotherapy. This attitude reflects, at least in part, pathologists reluctance to utilize frozen tissue sections in immunohistochemical assays. Furthermore, the use of formalin fixed tissues in immunohistochemical assays suffers from the very limited availability of anti-HLA Class I mAb staining formalin fixed tissues. To overcome these limitations, this proposal aims at i) developing mAb recognizing monomorphic determinants of HLA-A, B and C alleles and $\beta_2\text{-}\mu$ in formalin fixed tissues and ii) at determining whether staining with the developed anti-HLA Class I mAb of formalin fixed lesions from various types of cancer with an apparent different involvement of immunological events in their pathogenesis and in their clinical course generates clinically useful information. Furthermore, recent studies have shown that staining of malignant cells with mAb recognizing monomorphic determinants of HLA Class I antigens does not detect selective loss of HLA Class I allospecificities (4-7). There is scanty information about the frequency and clinical significance of selective loss of HLA Class I alleles in malignant lesions. Therefore, an additional goal of this proposal is to determine the frequency of the selective HLA-A2 antigen loss in various types of cancer and to assess its clinical significance.

The specific aims are:

- 1) To assess the frequency of HLA Class I antigen loss or downregulation in primary and metastatic lesions in various types of malignancies with an apparent different involvement of immunological events in their pathogenesis and in their clinical course. They include head and neck squamous cell carcinoma, breast carcinoma, carcinoma of the gastrointestinal tract, cervical carcinoma and melanoma. To this end, sections of formalin fixed lesions will be stained with mAb recognizing monomorphic determinants of HLA Class I heavy chains and with anti- $\beta_2\text{-}\mu$ mAb in immunoperoxidase reactions.
- 2) To assess the clinical significance of HLA Class I antigen loss or downregulation in primary and metastatic lesions in head and neck squamous cell carcinoma, in breast carcinoma, in carcinoma of the gastrointestinal tract, in cervical carcinoma and in melanoma by correlating level of HLA Class I antigen expression in lesions with their histopathological characteristics and with the clinical course of the disease.

- 3) To assess the frequency of selective HLA-A2 antigen loss or downregulation in primary and metastatic lesions in head and neck squamous cell carcinoma, in breast carcinoma, in carcinoma of the gastrointestinal tract, in cervical carcinoma and in melanoma by testing surgically removed frozen lesions with anti-HLA-A2 mAb in immunoperoxidase reactions.
- 4) To assess the clinical significance of HLA-A2 antigen loss or downregulation in primary and metastatic lesions in head and neck squamous cell carcinoma, in breast carcinoma, in carcinoma of the gastrointestinal tract, in cervical carcinoma and in melanoma by correlating the level of HLA-A2 antigen expression in lesions with their histopathological characteristics and with the clinical course of the disease.

Besides contributing to determine the clinical significance of abnormalities in HLA Class I antigens in various types of cancer, the outlined studies will generate reagents and methodology which will facilitate the screening and monitoring of patients with malignant diseases to be treated with T cell-based immunotherapy.

B. BACKGROUND AND SIGNIFICANCE

Altered MHC Class I antigen expression in tumors was first reported over a quarter of a century ago. MHC class I antigen loss was described in several mouse tumors and cell lines. It was soon realized that phenotypic alterations of MHC Class I antigen expression may permit tumor cells to avoid or survive attack by the immune system. This notion was supported by the enhanced growth of tumor cells which had downregulated MHC Class I antigen expression following transfection with antisense DNA and by the loss of tumorigenicity of aggressive MHC Class I-negative mouse tumor cells following transfection with MHC Class I genes. The latter results presumably reflect the induction of tumor associated antigen (TAA) specific, MHC Class I antigen restricted cytotoxic T lymphocytes (CTL) by the transfectants in the host. In spite of the relevance of these intriguing results for the development of immunotherapeutic approaches to cancer, interest in abnormal MHC Class I antigen expression by tumor cells waned in the 1980s. The loss of interest reflects, at least in part, the skepticism generated in the field by the realization that alien histocompatibility antigens described in a number of mouse tumors (8,9) were essentially technical artifacts caused by cross contamination of mouse strains and mouse cell lines. An additional negative influence on the development and continuation of this line of research was generated by the conflicting results about the clinical significance of HLA Class I antigen downregulation in malignant lesions in various types of cancer. In retrospect, the published conflicting results are not surprising given the heterogeneity of the patient populations investigated, the different role played by immunological events in the pathogenesis and clinical course of various types of cancer, the interference of a number of variables with the clinical significance of HLA Class I antigen downregulation and the differences in the characteristics of the mAb and in the sensitivity of the immunohistochemical assays used by the various investigators. However, renewed interest in MHC Class I antigen loss in tumors took place in the early 1990s with the realization of the crucial role played by MHC Class I antigens in the recognition of tumor cells by CTL (10) and with the emphasis on T cell-based immunotherapy for the treatment of human cancer (11,12).

Over the past two decades the availability of HLA-specific monoclonal antibodies (mAb) suitable for immunohistochemical staining and technical advancements in immunohistochemical staining techniques have allowed extensive analysis of HLA Class I antigen expression in cryopreserved tumors. These studies have conclusively shown that HLA Class I antigen downregulation occurs in a number of malignant lesions, although with marked differences in its frequency. The frequency of HLA Class I antigen downregulation is significantly ($p < 0.01$) higher in breast carcinoma and prostate carcinoma than in head and neck squamous cell carcinoma, lung carcinoma, colon carcinoma, cervical carcinoma and melanoma (13). Whether these differences reflect technical reasons, patient populations' heterogeneity and/or the different role played by HLA Class I antigens in various types of malignancies is not known.

These questions cannot be addressed by an individual laboratory in a comprehensive and systematic way. Therefore, they will be addressed by the working group we have organized, utilizing standardized reagents and methodology to analyze HLA Class I antigen expression in malignant lesions. Furthermore, the working group will investigate the clinical significance of HLA Class I antigen abnormalities in malignant lesions, since only limited and at times conflicting evidence is available in this regard. In a limited number of patients HLA Class I antigen downregulation in metastatic lesions appears to have a negative impact on the outcome of T cell-based immunotherapy of malignant diseases, since reduction or loss of HLA Class I antigen expression in metastatic lesions is associated with disease progression (14) or with recurrence of the disease in patients treated with T cell-based immunotherapy (2,3). In spite of these results, testing of surgically removed malignant lesions for HLA Class I antigen expression has not become a routine assay to evaluate patients with malignancies, even those to be enrolled in trials of T cell-based immunotherapy. This attitude reflects, at least in part, the pathologists' reluctance to utilize frozen tissue sections in immunohistochemical assays with anti-HLA Class I mAb. Frozen tissue sections have to be used in these assays, since only very few anti-HLA Class I mAb have been found to stain formalin fixed tissues in immunohistochemical assays. To the best of our knowledge, only mAb HC-10 and mAb HA-2 which recognize distinct determinants on heavy chains of some, but not all HLA Class I alleles (15,16) and only the anti- $\beta_2\text{-}\Phi$ mAb L368 (17) stain formalin fixed tissues in immunohistochemical reactions. Therefore, mAb HC-10 and mAb HA-2 may not detect all the HLA Class I alleles expressed in a tissue section and may generate false negative results. To overcome these limitations, we plan to develop mAb which recognize distinct monomorphic determinants expressed on all HLA-A, B and C antigens. Furthermore, we plan to develop mAb which recognize a determinant of $\beta_2\text{-}\Phi$ distinct from that recognized by mAb L368. The latter mAb recognizes a determinant which involves residues 38, 44 and 45 of $\beta_2\text{-}\Phi$ for its expression (18). We believe that mAb recognizing distinct determinants on each of the two subunits of HLA Class I antigens should be used to analyze the expression of HLA antigens in tissues, since lack of detection of an antigenic determinant does not always reflect loss of the corresponding molecule. The available mAb and those we plan to develop will be utilized by the collaborating laboratories to determine whether i) staining of formalin fixed tissue sections provides reliable information about the expression of HLA Class I antigens in lesions from various types of cancer with an apparent different involvement of immunological events in their pathogenesis and in the clinical course of the disease and ii) whether HLA Class I antigen downregulation in various types of malignancies has a clinical significance. The reactivity of anti-HLA Class I mAb with formalin fixed tissues will facilitate studies to address these questions, since it provides the opportunity to perform retrospective studies, utilizing collections of pathological lesions from patients with detailed information about the clinical course of the disease.

Recent studies with a limited number of malignant lesions have suggested that selective loss of HLA Class I alleles is frequent in malignant cells (13). The frequency of selective loss of HLA Class I alleles varies markedly among malignancies, being significantly higher in melanoma than in head and neck squamous cell carcinoma, breast carcinoma, lung carcinoma, colon carcinoma, cervical carcinoma and prostate carcinoma (for review, see 13). The reason(s) for these differences as well as the clinical significance of selective HLA Class I allele loss are not known. Immunohistochemical staining of malignant lesions and FACS analysis of cell lines with mAb to monomorphic determinants of HLA Class I antigens do not detect selective loss of a HLA Class I allele (4-7). Undetected selective loss of a HLA Class I allele may account for the unexpected poor prognosis of the disease in patients with high expression of HLA Class I antigens in primary and/or metastatic lesions, as measured by staining with mAb to monomorphic determinants of HLA Class I antigens (19). Furthermore, selective loss of a HLA Class I allospecificity is likely to have a negative impact on the efficacy of T cell-based immunotherapy which utilizes the lost HLA Class I allele as a restricting element. Therefore, we plan to investigate the frequency of selective loss of HLA-A2 alleles in primary and metastatic lesions in various types of cancer utilizing standardized methodology. The malignant diseases include head and neck squamous cell carcinoma, breast carcinoma, carcinoma of the gastrointestinal tract, cervical carcinoma and melanoma. Furthermore, we will assess the clinical significance of HLA-A2 antigen downregulation in these

malignancies by correlating its level of expression in surgically removed lesions with their histopathological characteristics and with the clinical course of the disease. We have selected the HLA-A2 allele as a model for our studies, since this allele has a high frequency in the population, therefore facilitating the recruitment of patients to the study. Furthermore, the HLA-A2 allele has been shown to present TAA derived peptides to CTL (20-22). Lastly, mAb which recognize distinct determinants of HLA-A2 antigens in frozen tissue sections have already been developed by us and are available for the study. We will use frozen tissue sections in these investigations, because the expression of polymorphic determinants of HLA Class I antigens requires the association of HLA Class I heavy chains with β_2 -m. Such an association is lost in formalin-fixed tissues because of the denaturing conditions of the fixation procedure.

In summary, the proposed studies address important questions related to the clinical significance of total or selective HLA Class I antigen(s) downregulation or loss in malignant lesions. The proposed studies have several unique aspects. First, the participation of many laboratories with an excellent track record in the area of HLA Class I antigen abnormalities in malignant cells will provide the opportunity to analyze a large number of lesions with well defined histopathological characteristics from patients with known clinical history utilizing standardized methodology and reagents. Therefore, the outlined investigations are expected to provide conclusive evidence about the frequency and clinical significance of HLA Class I antigen abnormalities in malignant lesions. Furthermore, the proposed in conjunction with the anti-HLA Class I mAb staining formalin fixed tissues to be developed in the course of this grant period will assess for the first time the validity of immunohistochemical staining of formalin fixed tissue sections to evaluate HLA Class I antigen abnormalities in malignant lesions. The information resulting from the outlined studies in conjunction with the anti-HLA Class I mAb staining formalin fixed tissues to be developed during the first year of the requested grant period will have a major impact on the introduction of the routine evaluation of HLA Class I antigen expression in malignant lesions in histopathology laboratories. Lastly, the proposed studies will evaluate for the first time the frequency and clinical significance of the selective loss of HLA-A2 antigens in malignant lesions. If the proposed studies yield meaningful results, what has been learnt from these studies can be applied to characterize the selective loss of other HLA Class I allospecificities in malignant lesions.

C. Preliminary Results

C.1. Identification of anti-HLA-A2 mAb recognizing distinct determinants of HLA-A2 antigens. The mAb CR11-351, HO1, HO-2, HO-3, HO-4, HO-5 and KS1 have been shown with immunochemical assays to be specific for HLA-A2 antigens. To identify mAb recognizing distinct determinants of HLA-A2 antigens, the seven mAb were tested in binding assays with a panel of 68 variant HLA-A2 molecules. The latter differ from HLA-A2.1 allospecificity by single amino acid substitutions in the α_1 and α_2 domains. The determinants recognized by mAb HO-1, HO-3 and HO-4 were not disrupted on any of the variants tested. The determinant recognized by mAb CR11-351 was mapped to positions 142, 145 and 149 in the α_2 domain, that recognized by mAb HO-5 to position 149 in the α_2 domain, that recognized by mAb KS1 to position 127 in the α_2 domain and that recognized by mAb HO-2 to positions 58 and 62 in the α_1 domain (Table I). On the basis of these results mAb HO-2 and a mAb which recognizes a determinant mapped to the α_2 domain of HLA-A2 antigen will be used to measure the expression of HLA-A2 antigens in tissues.

TABLE I
MAPPING OF DETERMINANTS RECOGNIZED BY ANTI-HLA-A2 mAb

mAb	domain	residue ^a
CR11-351	α_2	142,145,149
HO-2	α_1	58,62
HO-5	α_2	149
KS1	α_2	127

^aresidue involved in the expression of the antigenic determinant recognized by mAb

C.2. Organization of the HLA and cancer component for the 13th International Histocompatibility Working Group. In a meeting in Seattle, WA in September 1997 with other chairmen of the components of the 13th Histocompatibility Working Group the strategy to organize the HLA and cancer component of the workshop and to recruit participating laboratories was designed. The first investigators meeting was held in Granada, Spain in April 1998 during a symposium on HLA antigen abnormalities in cancer. During this meeting it was decided to focus on a limited number of well defined questions. For this reason, a limited number of malignancies was selected. In addition, it was decided to focus a major component of the workshop on the evaluation of formalin fixed tissues as a substrate to measure the expression of HLA class I antigens in malignant lesions. It is expected that the information resulting from these studies will have a major impact on the introduction of testing of patients for expression of HLA antigens in their malignant lesions as a criterion to evaluate patients with malignancies and as a criterion to select patients to be treated with T cell-based immunotherapy. To organize and monitor the activities of the workshop an organizing committee was formed. Its components are listed in Table II.

Table II. HLA and Cancer Advisory Committee

Sir Walter Bodmer	Hertford College, Oxford, United Kingdom
Dominique Charron	Institute Cordeliers, Paris
Renee Fauchet	Centre Hospitalier Regional et Universitaire de Rennes, France
F. Garrido	Hospital Virgen de las Nieves, Universidad de Granada, Spain
F. M. Marincola	Surgery Branch, NCI, NIH, Bethesda

To facilitate the exchange of information and reagents among participating laboratories, coordinators were appointed for three regions. They are S. Ferrone (Department of Immunology, Roswell Park Cancer Institute, Buffalo, NY), for laboratories in the USA, F. Garrido (Dpto. de Análisis Clínicos e Inmunología Hospital Virgen de las Nieves, Universidad de Granada, Granada, Spain) for laboratories in Europe and B. Tait (Tissue Typing Laboratory, Royal Melbourne Hospital, Parkville, Australia) for laboratories in South East Asia. The malignancies to be investigated were selected. The activities in each

disease are coordinated by the investigators listed in Table III. The laboratories recruited thus far to the workshop are listed in Table IV.

TABLE III
SELECTED MALIGNANCIES FOR THE WORKSHOP

Malignancy	Coordinator
Head and neck carcinoma	Manita Feenstra Department of Pathology University Hospital Utrecht Academisch Ziekenhuis Utrecht Heldelberglaan 100 3508 GA Utrecht The Netherlands
Breast carcinoma	Maria Worsham Department of Pathology Henry Ford Hospital Detroit, MI 48202 (USA)
Colon carcinoma	Federico Garrido Dpto. de Análisis Clínicos e Inmunología, Hospital Virgen de las Nieves Universidad de Granada Granada, Spain
Cervical cancer	Peter Stern Department of Immunology Christie CRC Research Centre Paterson Inst. for Cancer Research Christie Hospital NHS Trust Wilmslow Road Manchester M20 4BX United Kingdom
Melanoma	Catherine Stavropoulos-Giovas National Tissue Typing Center Genteal Hospital of Athens Athens, Greece

TABLE IV

PARTICIPATING LABORATORIES

S.H. Chan, WHO Immunology Centre, Faculty of Medicine, National University of Singapore, Kent Ridge, Singapore
R. Dawkins, Centre for Molecular Immunology and Instrumentation, Subiaco, Western Australia, Australia
C. Demanet, Academic Hospital, Brussels Free University, HLA-Laboratory, Blood Transfusion Center, Jette, Belgium,
M. Feenstra, Department of Pathology, University Hospital Utrecht, Utrecht, The Netherlands
A. Frey, Department of Cell Biology, New York University Medical School, New York, NY
F. Garrido, Dpto. de Análisis Clínicos e Inmunología Hospital Virgen de las Nieves, Universidad de Granada, Granada, Spain
D. Godelaine, Ludwig Institute for Cancer Research, Brussels, Belgium
C. Gordoeky, Department of Immunogenetics, Indre, Mexico
S. Guo, Research Laboratory of Immunology, Hunan Medical University, Hunan, P.R. China
N. Joshi, Immunology Division, Cancer Research Institute, Parel, Mumbai, India
A. Knuth, Medizinische Klinik, Hämatologie-Onkologie, Frankfurt, Germany
C. Landry, Ludwig Institute for Cancer Research, Brussels, Belgium
C. Leelayuwat, Department of Clinical Immunology, Khon Kaen University, Khon Kaen, Thailand
M. Maio, Centro di Riferimento Oncologico, Aviano, Italy
F. M. Marincola, Surgery Branch, NCI, NIH, Bethesda, MD
N.K. Mehra, Dept Histocompatibility & Immunogenetics, All India Institute of Medical Sciences, Ansari Nagar, New Delhi
B. Munkhbat, National Institute of Health, Mongolian Medical University, Ulaanbaatar, Mongolia
P.G. Natali, Immunology Laboratory, Regina Elena Cancer Institute, Rome, Italy
E. Naumova, University Hospital Alexandrovska, Division of Clinical and Transplantation Immunology, Sofia, Bulgaria
T.P.P. Phan, Central Biomedical Laboratory, Hanoi Medical School, Hanoi, Vietnam
T. Rajkumar, Department of Molecular Oncology, Cancer Institute, Chennai, Madras, India
D. Speiser, Ludwig Institute for Cancer Research, Division of Clinical Onco-Immunology, Luusanne, Switzerland
C. Stavropoulos-Giovas, Genteil Hospital of Athens, National Tissue Typing Center, Athens, Greece
P. Stern, Dept Immunology, Christie CRC Research Centre, Paterson Institute for Cancer Research, Manchester, UK
B.E. Tait, Victorian Transplantation and Immunogenetics Service, Rotary Bone Marrow Research Building, Royal Melbourne Hospital, Tissue Typing Laboratory, Parkville, Victoria, Australia
M. Tilanus, University Hospital Utrecht, Department of Pathology, Utrecht, The Netherlands
S. Vaidya, Tissue Antigen Laboratory, The University of Texas Medical Branch, Galveston, TX
M.J. Worsham, Department of Pathology, Henry Ford Hospital, Detroit, MI
P. Zheng, Department of Pathology, The Ohio State University Medical Center, Columbus, OH
L. Zheng, HLA Immunology Laboratory, North Taiping Hospital, Beijing, P.R. China

C.3. Development of anti- $\beta_2\Phi$ mAb recognizing determinants distinct from that identified by mAb L368. BALB/c mice have been injected subcutaneously with peptides KNGERIEKVEHS and EFTPTEKDEYAC corresponding to residues 52-63 and 80-91 of $\beta_2\Phi$ a (50 Φ g of each peptide/injection). Each peptide was conjugated to a carrier and administered with an adjuvant, as described in D.II.1.A.a, D.II.1.A.b. Sera harvested from mice following four immunizations have been found to react with $\beta_2\Phi$ in ELISA and in Western blotting. Splenocytes from the immunized mice will be used to generate hybridomas.

D. Research Design and Methods

The working group we have organized is expected to provide conclusive evidence about the frequency and clinical significance of HLA Class I antigen abnormalities in malignant diseases, since a standardized methodology will be utilized and a large number of well characterized malignant lesions from patients with detailed information about the clinical course of the disease will be analyzed.

The collaborative work among the participating laboratories has been organized as follows:

- i) mAb to be used in immunohistochemical staining of tissues will be developed and characterized by S. Ferrone.
- ii) large amounts of mAb will be prepared and characterized in their activity and their specificity. Furthermore, each participating laboratory will monitor the activity and specificity of the received mAb preparations by testing with reference cell lines and tissue sections.
- iii) staining of pathological lesions with mAb will be performed by each participating laboratory utilizing a common standardized immunoperoxidase staining protocol. Results will be collected and sent to S. Ferrone. Furthermore, random stained tissue sections will be exchanged among laboratories in order to monitor the interlaboratory variability of results.
- iv) the results will be analyzed by the core Biostatistics and the analyses distributed to the participating laboratories.

D.I. Materials

D.I.1. Cell lines. A panel of HLA typed cell lines is maintained in our laboratory. Additional HLA typed cell lines will be obtained from Core C.

D.I.2. Tissues. Primary malignant lesions, autologous metastatic lesions, autologous normal tissues and autologous lymphocytes from patients undergoing surgery are already available or will be collected in participating laboratories or will be collected by the participating laboratories from patients undergoing surgery.

D.I.3. mAb. Hybridomas secreting mAb recognizing monomorphic determinants of HLA Class I antigens, anti- $\beta_2\Phi$ mAb and mAb recognizing distinct determinants of HLA-A2 antigen have been developed in our laboratory. The hybridomas secreting the mAb HA-A2 and HC-10 (15) which recognize determinants of heavy chains of many, but not all HLA Class I alleles in formalin fixed tissues, and the hybridoma secreting the mAb L368 (17) which recognizes $\beta_2\Phi$ in formalin fixed tissues have been made available to us. Large batches of mAb will be prepared from these hybridomas. They will be characterized in their specificity and activity. Standardized mAb preparations will be distributed to the participating laboratories.

D.2. Research Design

Because of space limitations the methodology is described briefly, since it has already been utilized by the applicant, as shown by the previously published papers which are enclosed.

D.2.1. To assess the frequency of total HLA Class I antigen loss or downregulation in primary and metastatic lesions in head and neck squamous cell carcinoma, in breast carcinoma, in carcinoma of the gastrointestinal tract, in cervical carcinoma and in melanoma by testing formalin fixed, paraffin embedded tissue sections with mAb recognizing distinct monomorphic determinants of HLA Class I heavy chains and distinct determinants of $\beta_2\Phi$.

Background, Rationale and General Approach

Thus far total HLA Class I antigen loss or downregulation in malignant lesions has been assessed by testing frozen tissue sections with mAb to monomorphic determinants of HLA Class I antigens and with anti- $\beta_2\Phi$ mAb. Although these studies have yielded valuable information, assessment of HLA Class I antigen expression in malignant lesions has not become a routine procedure to evaluate patients with malignancies, especially those to be enrolled in trials of T cell-based immunotherapy, mainly because of pathologists reluctance to utilize frozen tissue sections in immunohistochemical assays. To the best of

our knowledge, the only anti-HLA Class I mAb to stain formalin fixed tissue sections are mAb HA-2 (15), HC-10 (15) and L368 (17). mAb HA-2 and HC-10 recognize distinct determinants expressed on the heavy chains of many, but not all HLA Class I alleles. Therefore, staining of formalin fixed tissue sections with mAb HA-2 and HC-10 may underestimate the expression of HLA Class I alleles in malignant lesions or even yield false negative results. mAb L368 recognizes one determinant of $\beta_2\Phi$. Since lack of staining of a lesion with one mAb does not always reflect loss of the molecule expressing the tested determinant, assessment of the expression of $\beta_2\Phi$ in lesions by staining only with mAb L368 may yield false negative results. To overcome these limitations, we plan to develop mAb which recognize distinct monomorphic determinants on the heavy chains of all HLA Class I alleles. In addition, we plan to develop anti- $\beta_2\Phi$ mAb which recognize determinants distinct from that defined by mAb L368 in formalin fixed tissues. The latter mAb recognizes a determinant which involves residues 38, 44 and 45 $\beta_2\Phi$ (18). We plan to use these reagents to assess the frequency of total HLA Class I antigen loss or downregulation in the mentioned malignancies utilizing formalin-fixed tissues. The results of these studies are expected to have a major impact on the introduction in routine pathology laboratories of the evaluation of HLA Class I antigen expression in malignant lesions from patients, especially those to be treated with T cell-based immunotherapy.

Methods of Procedure

D.II.1.A. Development and characterization of mAb which recognize monomorphic determinants expressed on the heavy chains of all HLA Class I alleles in formalin fixed tissues.

D.II.1.A. a) Immunogens. Twelve-14 amino acid long peptides derived from the sequences in α_3 domains of heavy chains common to all HLA Class I alleles with the addition of a carboxyterminal cysteine residue are purchased from Yong Seng Trading Co., New York, NY. Peptides are coupled to maleimideactivated keyhole limpet hemocyanin (KLH) and to KLH via 1-ethyl-3-[3'-dimethylaminopropyl]-carbodiimide hydrochloride (Pierce, Rockford, IL) followed by purification on a Sephadex G-25 column (Pharmacia Fine Chemical, Piscataway, NJ).

D.II.1.A. b) Immunization schedule. Eight to 12 week old female BALB/c mice are primed with a subcutaneous injection of KLH conjugated peptide (50 Φ g/injection), emulsified in complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO). Mice are boosted at two week intervals with 50 Φ g of the same immunogen emulsified in incomplete Freund's adjuvant. Mice are bled at two week intervals. Mice which develop high titer antibodies reacting with HLA Class I heavy chains in Western blotting are boosted intravenously with 50 Φ g of the immunogen 3-5 days before splenocytes are harvested.

D.II.1.A. c) Generation of anti-HLA Class I mAb secreting hybridomas. Splenocytes are fused to mouse myeloma cells P3-X-63-Ag8.653. Hybridization and subcloning are performed using standard techniques (23). Subtyping of mAb is performed with the Isostrip kit (Boehringer-Mannheim Corp, Indianapolis, IN) following the manufacturers instructions. Hybridomas are screened for reactivity with the immunizing peptide in ELISA with a peptide coated plate, following the methodology we have previously utilized (24).

D.II.1.A. d) Characterization of the specificity of anti-HLA Class I mAb. mAb are tested in ELISA with cultured B lymphoid cells with different HLA phenotypes. We do not expect reactivity of the generated mAb with viable cells, since they are not likely to react with $\beta_2\Phi$ associated HLA Class I heavy chains. However, this possibility cannot be excluded since a few mAb have been found to react with both $\beta_2\Phi$ associated and $\beta_2\Phi$ free HLA Class I heavy chains (25-27). mAb will be tested for reactivity with $\beta_2\Phi$ free HLA Class I heavy chains in Western blotting, utilizing peroxidase-conjugated

goat anti-mouse IgG antibodies (Boehringer-Mannheim Corp.) and Enhanced Chemiluminescence substrate (Amersham, Arlington Heights, IL) as a detection reagent.

The distribution of the determinants recognized by the developed mAb on HLA Class I alleles will be assessed utilizing detergent solubilized lysates of cultured human B lymphoid cells with the appropriate HLA phenotypes. HLA Class I alleles are separated by one-dimensional iso-electric focusing (1D-IEF) (28) and then tested with mAb in immunoblotting (29). The mAb which are found to react with all HLA Class I alleles will also be tested for their ability to deplete from lymphoid cell extracts all HLA Class I heavy chains recognized by a rabbit anti-HLA Class I heavy chain antiserum (30).

mAb which recognize monomorphic determinants on all HLA Class I alleles will be tested for their ability to stain formalin fixed tissues. The staining patterns of formalin fixed tissue sections with the newly developed mAb will be compared to those of frozen tissue sections stained with known anti-HLA Class I mAb. Those newly developed mAb which stain formalin fixed tissues with a pattern compatible with the detection of HLA Class I antigens in formalin fixed tissues will be tested in crossblocking experiments to determine whether they recognize distinct determinants.

D.II.1.B. Development and characterization of anti- $\beta_2\text{-}\Phi$ mAb which stain formalin fixed tissues.

D.II.1.B. a) Immunogens. The peptide KNGERIEKVEHS corresponding to residues 52-63 of $\beta_2\text{-}\Phi$ with the addition of a carboxyterminal cysteine residue and the peptide EFTPTEKDEYAC corresponding to residues 80-91 of $\beta_2\text{-}\Phi$ are purchased from Yong Seng Trading Co. Peptides are coupled to a carrier as described in D.II.1.A. a.

D.II.1.B. b) Immunization schedule. The immunization schedule described in D.II.1.A.b. will be utilized.

D.II.1.B. c) Generation of anti- $\beta_2\text{-}\Phi$ mAb secreting hybridomas. The methodology indicated in D.II.1.A.c will be utilized for these experiments.

D.II.1.B. d) Characterization of the specificity of anti- $\beta_2\text{-}\Phi$ mAb. The newly developed mAb will be tested for differential reactivity in ELISA with cells which express or do not express $\beta_2\text{-}\Phi$. Furthermore, the newly developed mAb will be tested for reactivity with $\beta_2\text{-}\Phi$ in Western blotting utilizing peroxidase-conjugated goat anti-mouse IgG antibodies (Boehringer-Mannheim Corp.) and Enhanced Chemiluminescence substrate (Amersham, Arlington Heights, IL) as a detection reagent. The newly developed anti- $\beta_2\text{-}\Phi$ mAb will also be tested for their ability to immunodeplete lymphoid cell extracts of $\beta_2\text{-}\Phi$ recognized by a rabbit anti- $\beta_2\text{-}\Phi$ antiserum.

The newly developed anti- $\beta_2\text{-}\Phi$ mAb will be tested for their ability to stain formalin fixed tissues. The staining patterns of formalin fixed tissue sections with the newly developed mAb will be compared with that obtained with the already characterized anti- $\beta_2\text{-}\Phi$ mAb L368. The newly developed anti- $\beta_2\text{-}\Phi$ mAb which stain formalin fixed tissues will be tested in crossblocking experiments with mAb L368 to determine whether they recognize different antigenic determinants from that defined by mAb L368.

D.II.1.C. Assessment of the frequency of total HLA Class I antigen loss or downregulation in head and neck squamous cell carcinoma, in breast carcinoma, in carcinoma of the gastrointestinal tract, in cervical carcinoma and in melanoma utilizing formalin fixed lesions. Formalin fixed sections of primary and metastatic lesions will be tested in immunoperoxidase reactions with mAb recognizing two distinct monomorphic determinants expressed on HLA Class I heavy chains and two distinct determinants expressed on $\beta_2\text{-}\Phi$. The use of at least two mAb for each HLA Class I subunit will allow us to determine whether malignant cells have lost the expression of one determinant or of HLA Class I molecules. The immunoperoxidase staining of formalin fixed, paraffin embedded tissue sections will be

performed as follows. Four um thick sections are deparaffinized with xylene and rehydrated. After preincubation with 5% normal goat serum for 30 min, sections are incubated overnight at 4 °C with a mAb preparation. Following three washes with PBS, tissue sections are incubated with biotinylated anti-mouse IgG xenoantibodies for 30 min at room temperature and then allowed to react with the avidin biotin peroxidase complex for 60 min at room temperature. A positive reaction is detected by the development of peroxidase staining using diaminobenzidine as the substrate. Tissue sections are counterstained with a Giemsa solution and mounted with Eukit. Control reactions are performed replacing the first mAb with a myeloma protein.

The percentage of stained malignant cells and staining intensity will be estimated independently by two observers. It has been our experience that variations in the percentage of stained cells enumerated by two experienced observers is less than 10%. The average percentage will be calculated and utilized for further analysis. When questions arise about the nature of the cells stained by anti-HLA mAb, the nature of the malignant cells will be assessed by staining with mAb recognizing a tumor associated antigen (TAA) characteristic of the tumor being investigated, whenever mAb with such a specificity are available. The results will be classified as positive, heterogeneous and negative, when the percentage of stained tumor cells in the entire lesions is more than 75, between 75 and 25, and less than 25, respectively. Staining intensity will be scored as - (absent), ∇ (dull) and + (bright). The staining intensity of adjacent normal structures (i.e. lymphoid and endothelial cells) will be used as an internal control to evaluate the staining intensity of malignant cells.

To minimize the variability among laboratories the same batch of mAb will be distributed to all the participating laboratories. Each laboratory will be asked to test the activity of each antibody preparation with a selected cell line and with two selected tissue sections. Furthermore, stained tissue sections chosen at random will be exchanged among laboratories and read blindly. Visits among laboratories will be encouraged in order to facilitate exchange of information and comparisons of staining patterns.

Whenever sufficient tissue is available and/or a cell line has been derived from a lesion with total HLA Class I antigen loss or downregulation, the results of immunoperoxidase staining will be corroborated by SDS-PAGE analysis of antigens immunoprecipitated from extrinsically and/or intrinsically radiolabelled cells by anti-HLA Class I mAb and by Western blotting testing of cell extracts with anti-HLA Class I heavy chain mAb and with anti- $\beta_2\text{-}\Phi$ mAb.

We expect that at least 200 primary and 200 metastatic lesions of each type of listed malignancy will be evaluated by the participating laboratories. To assess the inter-laboratory variability, univariate analyses will be performed for each participating laboratory. For each malignancy, the results obtained by the various laboratories will be compared using ANOVA for percentage of stained malignant cells and Fishers exact test for percentages of positive, heterogeneous and negative results. Univariate analyses such as means (sd) of the percentages of stained malignant cells and percentages of positive, heterogeneous and negative or absent, dull and bright results will be first performed to assess the frequency of HLA class I antigen loss or downregulation in primary and metastatic lesions in each malignancy. These results will be compared among different malignancies using ANOVA for the percentages (or its transformations) of stained cells or the Fisher's exact test for the positive/heterogeneous/negative, or absent/dull/bright results.

D.II.2. To assess the clinical significance of HLA class I antigen loss or downregulation in primary and metastatic lesions in head and neck squamous cell carcinoma, in breast carcinoma, in carcinoma of the gastrointestinal tract, in cervical carcinoma and in melanoma.

Background, Rationale and General Approach

To assess the clinical significance of HLA Class I antigen loss or downregulation in primary and metastatic lesions in the above listed malignancies, the level of HLA Class I antigen expression will be

correlated with the histopathological characteristics of the lesions and with the clinical course of the disease.

It is our working hypothesis that HLA Class I antigen loss or downregulation is associated with markers of poor prognosis. Differences may be found in the association in the various types of malignancies we plan to investigate. These differences may reflect the different role played by TAA-specific, HLA Class I antigen restricted CTL responses in the various types of malignancies.

Methods of Procedure

D.II.2.A. Assessment of HLA Class I antigen loss or downregulation in malignant lesions. HLA Class I antigen expression in primary and metastatic lesions will be measured by testing in immunoperoxidase reaction with anti-HLA Class I heavy chain mAb and with anti $\beta_2\text{-}\Phi$ mAb. The reactions are evaluated in terms of percentage of stained malignant cells and staining intensity. The results are expressed as described in D.II.1.C.

D.II.2.B. Clinical relevance of HLA Class I antigen loss or downregulation in malignant lesions. Relationship of the levels of HLA Class I antigen expression in tumor cells and the histopathological features of lesions such as grade and stage, anatomic site of lesions, and metastases in case of metastatic lesions will be analyzed using ANOVA for percentages (or its transformations if non-normal) of the stained cells and using the Fishers exact test for the positive/heterogeneous/negative, or absent/dull/bright results. The prognostic significance of HLA Class I antigen loss or downregulation as they relate to disease-free survival (time to recurrence from diagnosis of the disease), overall survival (time to death from diagnosis of the disease, or time from first diagnosis of a metastasis to death) will be assessed by using the logrank test and the Kaplan-Meier curve (31). In addition, the Cox proportional hazards regression (32) will be used for multivariate analyses to account for possible confounding variables such as tumor burden and performance status, stage and grade of the tumor etc. Backward stepwise Cox regression will also be performed to identify independent predictors for disease-free survival and overall survival. The assumptions of proportional hazards will be verified empirically. If this assumption is violated, an interaction term with time will be added to the regression model.

At least two blocks of tissue of each lesion will be tested to minimize sampling errors and technical failures. The proportion of malignant cells stained may be markedly different (more than 20%) between the two blocks studied; in our previous studies (19), this has occurred in about 10% of the lesions tested. In such cases, if tissue is available, we will test two additional sections and then we will use the mean of the values in our analyses. If tissue is not available, the case will not be used in additional analyses. The level of HLA Class I antigens may be heterogeneous between primary and metastatic lesions as well as among multiple metastases removed from different anatomic sites (19). Therefore, in our studies, we will analyze separately the level of HLA Class I antigens in primary and metastatic lesions. The resulting information will assess the relative significance of the level of these antigens in primary and metastatic lesions. In the analysis of the level of HLA Class I antigens in multiple autologous metastases, we will test three approaches: i) we will use the mean of the values obtained in all the metastases from each patient investigated, ii) we will use the mean of the values obtained in the majority (at least 80%) of the metastases tested from each patient and iii) we will analyze separately the mean values obtained in distinct sets of metastases (i.e., visceral towards cutaneous metastases; metastases which appear concomitantly as compared with metastases which appear at different times). On the basis of the results obtained in the preliminary studies, we will decide which approach is most informative. If significant inter-laboratory variability is found (see section D.II.1.C.), an additional random effect term (such as gamma frailty) will be added in the Cox regression model to control for inter-laboratory variability.

We expect that at least 200 primary and 200 metastatic lesions of each type of listed malignancy will be tested by the participating laboratories. For this sample size, for an alpha level of 0.05 and power of 0.80 based on two-sided logrank test, the detectable relative risk for disease recurrence or death for

different possible percentages of HLA Class I antigen loss or downregulation and for different possible number of events, i.e. recurrence or death is shown in Table V.

TABLE V
DETECTABLE RELATIVE RISKS OF RECURRENCE OF DEATH FOR
DIFFERENT PERCENTAGES OF HLA CLASS I ANTIGEN LOSS AND
DIFFERENT NUMBER OF RECURRENCES OR DEATHS

HLA Class I antigen loss or downregulation	Number of events ^a			
	30	50	70	90
20%	3.7	2.7	2.3	2.1
30%	3.1	2.4	2.1	1.9
40%	2.9	2.9	2.0	1.8
50%	2.8	2.2	2.0	1.8

^aDisease recurrences or deaths

D.II.3. To assess the frequency of selective HLA-A2 antigen loss or downregulation in head and neck squamous cell carcinoma, in breast carcinoma, in carcinoma of the gastrointestinal tract, in cervical carcinoma and in melanoma by testing frozen tissue sections with mAb recognizing distinct determinants of HLA-A2 antigens.

Background, Rational and General Approach

Recent studies with a limited number of malignant lesions (4,5) and of cell lines (6,7) have shown that selective loss of HLA Class I allospecificities is not associated with detectable changes in the staining by mAb to monomorphic determinants of HLA Class I antigens. It is our working hypothesis that selective loss of HLA Class I allospecificities may cause resistance of malignant cells to lysis by TAA-specific CTL which utilize the lost HLA Class I allospecificity as a restricting element. Furthermore, it is our working hypothesis that selective loss of a HLA Class I allele may underlie the unexpected poor clinical course of the disease in patients with malignancies without detectable abnormalities in the staining of malignant lesions by mAb which recognize monomorphic determinants of HLA Class I antigens.

To test the validity of our hypothesis we have selected HLA-A2 antigens. The reasons for selecting this allospecificity for our studies are that i) the expression of HLA-A2 antigens in about 40% of patients with malignancies will facilitate the accrual of a sufficient number of patients to perform a statistical analysis of the results; ii) the available information indicates that HLA-A2 antigens are selectively lost in some malignant lesions with a frequency of at least 20% (4,5); iii) HLA-A2 antigen has been shown to be a restricting element in the interaction of some types of tumor cells with TAA specific CTL (20-22) and iv) a panel of anti-HLA-A2 mAb which recognize distinct determinants we have mapped on HLA-A2 molecules is available to us.

Methods of Procedure

D.II.3.A. To assess the frequency of the selective HLA-A2 antigen loss or downregulation in malignant lesions without detectable abnormalities in the staining by mAb to monomorphic determinants of HLA Class I antigens. Frozen sections of primary and metastatic lesions from HLA-A2 positive patients will be tested in the immunoperoxidase reaction with mAb recognizing monomorphic and locus restricted determinants of HLA Class I antigens and with mAb recognizing two distinct determinants of HLA-A2 antigens. The use of two anti-HLA-A2 mAb will allow us to distinguish between the possibilities that malignant cells have lost the expression of a polymorphic determinant or of molecules bearing the HLA-A2 allospecificity.

The immunoperoxidase staining will be performed as described in D.II.1.C. except for the use of frozen tissue sections. They will be incubated for 30 min at room temperature with PBS, pH 7.4, supplemented with 5% goat serum. Tissue sections will then be incubated with mAb and the reaction will be continued as described in D.II.1.C.

The percentage of stained malignant cells and staining intensity will be estimated independently by two observers. It has been our experience that variations in the percentage of stained cells enumerated by two experienced observers is less than 10%. The average percentage will be calculated and utilized for further analysis. When questions arise about the nature of the cells stained by anti-HLA Class I mAb, the nature of the malignant cells will be assessed by staining with mAb recognizing a TAA characteristic of the tumor being investigated, whenever mAb with such a specificity are available. The results will be classified as positive, heterogenous and negative, when the percentage of stained tumor cells in the entire lesion is more than 75, between 75 and 25, and less than 25, respectively. Staining intensity will be scored as - (absent), ∇ (dull) and + (bright). The staining intensity of adjacent normal structures (i.e. lymphoid and endothelial cells) will be used as an internal control to evaluate the staining intensity of malignant cells.

Whenever sufficient tissue and/or cell lines are available, the selective loss of HLA-A2 antigens by malignant cells will be confirmed by SDS-PAGE analysis of antigens immunoprecipitated by anti-HLA-A2 mAb from intrinsically and extrinsically radiolabelled malignant cells, by IEF of HLA Class I antigens immunoprecipitated from radiolabelled malignant cells by mAb recognizing monomorphic determinants on β_2 - Φ associated and β_2 - Φ free HLA Class I heavy chains and by oligonucleotide typing by sequence specific oligonucleotide probes (33-35). We have already used the three methods to define the HLA phenotype of melanoma cell lines, as published in a recent paper (36).

The HLA Class I phenotype of malignant cells will be compared to that of autologous peripheral blood lymphocytes. The latter will be determined utilizing the conventional complement dependent cytotoxic test and oligonucleotide typing by sequence specific oligonucleotide probes (33-35). Furthermore, in lesions stained with anti-HLA-A2, the staining of melanoma cells will be compared with that of surrounding normal cells, i.e. endothelial cells and lymphocytes.

We expect that a least 200 primary and 200 metastatic lesions will be tested for each type of listed malignancy by the participating laboratories. Similar analyses as those described in D.II.1.c to analyze the levels of HLA Class I antigens will be performed to analyze the inter-laboratory variability and the frequency of HLA-A2 antigen loss or downregulation in primary and metastatic lesions in each malignancy. The results will be compared among different malignancies.

D.II.4. To assess the clinical significance of the selective HLA-A2 antigen loss or downregulation in primary and metastatic lesions in malignancies with an apparent different involvement of immunological events in their pathogenesis and in the clinical course of the disease, i.e. head and neck squamous cell carcinoma, in breast carcinoma, in carcinoma of the gastrointestinal tract, in cervical carcinoma and in melanoma.

Background, Rationale and General Approach

To assess the clinical significance of the selective HLA-A2 antigen loss or downregulation in primary and metastatic lesions without in the above listed malignancies, the level of HLA-A2 antigen expression will be correlated with the histopathological characteristics of the lesions and with the clinical course of the disease. It is our working hypothesis that selective HLA-A2 antigen loss or downregulation may account for the poor clinical course of the disease in patients without abnormalities in the staining of their malignant lesions by mAb to monomorphic determinants of HLA Class I antigens, when HLA-A2 antigen is used as a restricting element by TAA specific CTL.

Methods of Procedure

D.II.4.A. Assessment of HLA-A2 antigen loss or downregulation in malignant lesions. HLA-A2 antigen expression in primary and metastatic lesions will be measured by testing frozen tissue sections in immunoperoxidase reactions with mAb recognizing distinct determinants of HLA-A2 antigens. The expression of HLA Class I antigens in the lesions will be monitored by staining with mAb to monomorphic and locus restricted determinants of HLA Class I antigens. Furthermore, as already mentioned in section D.II.3.A. whenever possible the selective loss of HLA-A2 antigens will be corroborated by SDS-PAGE analysis of antigens immunoprecipitated by anti-HLA-A2 mAb from radiolabelled malignant cells, by IEF of HLA Class I antigens immunoprecipitated by mAb to monomorphic determinants expressed on $\beta_2\text{-}\Phi$ associated and $\beta_2\text{-}\Phi$ free HLA Class I heavy chains and by oligonucleotide typing by sequence specific oligonucleotide probe (32-34).

D.II.4.B. Clinical relevance of selective HLA-A2 antigen loss or downregulation in malignant lesions.

The same analyses as those described in D.II.2.B. to analyze the clinical significance of HLA Class I antigen loss or downregulation will be performed to correlate the levels of HLA-A2 antigen in lesions with their histopathological features, anatomic site, and extent of metastases in case of metastatic lesions. Similar survival analyses as those described to analyze HLA Class I antigen loss or downregulation will be performed to correlate the levels of HLA-A2 loss or downregulation with disease-free survival, overall survival and time from first metastasis to death.

For a sample of 200 patients for each malignancy investigated, Table V in D.II.2.C. shows the detectable relative risk between patients with HLA-A2 antigen loss or downregulation and patients with no detectable HLA-A2 antigen loss or downregulation for risk of recurrence or death.

Program Aspects

This component will interact with the component HLA null alleles and DNA - serology equivalents to compare the frequency of HLA-A2 antigen loss in malignant cells and in normal cells and to compare the mutations underlying HLA-A2 antigen loss in normal and malignant cells. Furthermore, this component will interact with the component Biology of HLA-E, F and G to compare the expression of classical and non classical HLA Class I antigens in malignant cells. Furthermore, this component will continue to interact with the core Biostatistics for the statistical analysis of the results. In addition, this component will interact with the core Cell, null allele and gene bank to obtain HLA typed cell lines which will be utilized to characterize the specificity of the mAb to be developed. This component will also interact with the core SSOP and SBT typing and quality control for typing malignant cells and for identifying the molecular defect(s) in cells with abnormalities in HLA Class I antigen expression.

GENDER AND MINORITY

The studies proposed here will involve tumor samples submitted to surgical pathology. Gender and minority representation should reflect the overall demographics of patients referred to the treating hospital, and in some cases gender representation will reflect the specific tumors studied such as breast and cervical carcinoma. Diverse racial participation should be facilitated through multi-national collaboration.

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