MARKEDLY DECREASED EXPRESSION OF CLASS I HISTOCOMPATIBILITY ANTIGENS, PROTEIN, AND mRNA IN HUMAN SMALL-CELL LUNG CANCER

By AUSTIN DOYLE, W. JOHN MARTIN, KEIKO FUNA, ADI GAZDAR, DESMOND CARNEY, SUE ELLEN MARTIN, ILONA LINNOILA, FRANK CUTTITTA, JAMES MULSHINE, PAUL BUNN, and JOHN MINNA

From the NCI-Navy Medical Oncology Branch and the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, and Naval Hospital, Bethesda, Maryland 20814; the Department of Pathology, Uniformed Services Medical School, Bethesda, Maryland 20814; and the Department of Pathology, National Institutes of Health, Bethesda, Maryland 20814

Class I major histocompatibility complex (MHC)[1] antigens (HLA-A,B,C and β_2m [β_2-microglobulin]) are expressed on most nucleated human cells, with neurons and the mature trophoblast being the principal exceptions (1, 2). However, a marked deficiency in expression of these antigens has been noted in human embryonal carcinoma (3) and neuroblastoma cell lines (2), as well as isolated cell lines of lymphoid or epithelial malignancies (4, 5). These studies have shown that human and murine cell lines devoid of surface expression of class I histocompatibility antigens could lack peptide chain synthesis of either β_2m or the HLA-A,B,C antigen, since cells deficient in β_2m are unable to transport HLA proteins to the cell surface. In the F9 mouse embryonal carcinoma line, the defect in class I MHC antigens has been shown (6) to be a marked reduction in H-2 and β_2m mRNA transcription, which could be increased by incubating the cells in retinoic acid.

Because these molecules play an important role in immune cell recognition, the low or absent expression of HLA-A,B,C antigens in malignant tissues has been viewed with great interest for its implications in tumor growth and metastasis. In addition, rat cells transformed by the E1a region of the highly oncogenic adenovirus 12 have diminished expression of the heavy chain of the rat class I MHC, which continues to be expressed in cells transformed by the nononcogenic adenovirus 5 (7). Thus, tumors with absent class I MHC expression may contain an activated E1a-like product.

In this paper, we describe marked deficiencies of expression of HLA-A,B,C and β_2m on a panel of human small-cell lung cancer (SCLC) cell lines. In patients,

This work was supported in part by grant CA 33693 from the National Cancer Institute. Requests for reprints should be addressed to A. Doyle, University of Maryland Cancer Center, Rm. S9D08A, University of Maryland Hospital, 22 South Greene St., Baltimore, Maryland 21201.

[1] *Abbreviations used in this paper*: APUD, amine precursor uptake and decarboxylation; β_2m, β_2-microglobulin; BSA, bovine serum albumin; LNFP3, lacto-*N*-fucopentose III; MHC, major histocompatibility complex; NSCLC, non–small-cell lung cancer; PBS, phosphate-buffered saline; SCLC, small cell lung cancer; SSEA, stage-specific embryonic antigen.

SCLC exhibits rapid growth and early metastases, suggesting resistance to immune control. These deficits contrast SCLC with human lung cancers of other histologic types, including adenocarcinoma, epidermoid carcinoma, and large-cell carcinoma, which we have found to readily express class I antigens. These results indicate an important biological difference between SCLC and non–small-cell lung cancer (NSCLC) cells that may be related to differences in the clinical and molecular biologic behavior of these two categories of human lung cancer.

Materials and Methods

Cell Lines. The human SCLC lines were grown in our laboratory in RPMI 1640 (Gibco Laboratories, Grand Island, NY) with 10% heat-inactivated fetal bovine serum, as described previously (8, 9). Cell lines derived from classic human SCLC tumors were NCI-H209, H69, H146, H128, H60, H446, and H187. These cell lines are distinct by morphology, by a deletion of the short arm of the third chromosome, characteristic of SCLC (10), by production of bombesin and neuron-specific enolase, and by different patterns of reactivity to monoclonal antibodies developed in our laboratories (11). Biochemical and morphologic variants of SCLC included NCI-H82, H524, H526, and N417. Although derived from patients with SCLC, variant cell lines are characterized (12) by increased growth rate and colony-forming efficiency, lack of amine precursor uptake and decarboxylation (APUD) markers, radioresistance, and *myc* amplification, in contrast to classic SCLC. NSCLC lines used were NCI-H23 and H125 (adenocarcinoma), NCI-H157 (large cell), H292 (mucoepidermoid), A427 (epidermoid), and H226 (mesothelioma). Other tumor lines assayed were NCI-H78 and H102 (malignant T cell) and H234 (melanoma). Two B lymphoblastoid lines (H128BL, H209BL) derived from SCLC patients were also tested. The SCLC, malignant T cell, and B lymphoblastoid lines grew in suspension. The other lines grew attached to plastic tissue culture flasks and were scraped off with a rubber policeman. All cells were washed with phosphate-buffered saline (PBS) before use.

Monoclonal Antibodies. Monoclonal antibody W6/32 (Accurate Chemical & Scientific Corp., Westbury, NY) reacts weakly with an invariant determinant on the HLA chain of all HLA-A,B,C molecules and more strongly with the native two-chain molecule (13). Monoclonal antibodies H-0592 (Hybritech, Inc., La Jolla, CA) and 9456-SA (Bethesda Research Laboratories, Gaithersberg, MD) also react with invariant determinants of the HLA-A,B,C antigen. Monoclonal antibodies L368 (Becton Dickinson Monoclonal Antibody Center, Sunnyvale, CA), BBM.1 (American Type Culture Collection), and H-0602 (Hybritech, Inc.) react with β_2m. Radiobinding assays were done at an antibody concentration of 10 μg/ml in 1% bovine serum albumin (BSA). A conventional rabbit antisera specific for human β_2m was also used (Dako Corp., Santa Barbara, CA).

Radioimmunoassay. The radiobinding assay was performed in 96-well polyvinyl microtiter plates (Costar, Data Packaging, Cambridge, MA). Target cells were fixed at a density of 10^5 cells per well with 0.25% glutaraldehyde and then the wells were blocked with 1% BSA in PBS (11). The plates were incubated with antibody for 1 h, followed by affinity-purified rabbit anti-mouse hyperimmune serum for 1 h. All incubations were in PBS at room temperature. The detecting reagent used was 40,000 cpm of ^{125}I-labeled protein A (sp act, 30 μCi/μg). Purified mouse myeloma IgG2a (RPC5; Litton Bionetics, Inc., Kensington, MD) was used as a negative control.

Indirect Immunofluorescence Assay. Live cell suspensions were incubated under saturating conditions with monoclonal antibodies to HLA-A,B,C or β_2m determinants for 30 min at room temperature, washed, and incubated with a fluoresceinated goat anti-mouse secondary antibody (Becton Dickinson, Monoclonal Antibody Center) for 30 min at room temperature. The fluorescence intensity of 2–5 × 10^4 cells was analyzed in an Epics V cell sorter (Coulter Electronics, Inc., Hialeah FL). Control cells were incubated with a purified mouse myeloma IgG2A primary antibody and the same secondary antibody under identical conditions.

Immunoprecipitation. Cell lines were labeled metabolically for 12 h with [^{35}S]methionine (Amersham Corp., Arlington Heights, IL). 10 million cells from each line were labeled with 1 mCi of radionuclide. Cell lysates were prepared as described (14). For immunoprecipitation, we complexed monoclonal antibodies to protein A–Sepharose and used this complex to immunoprecipitate the cell lysates. Normal mouse sera was used as a negative control for each lysate. The precipitates were displayed by electrophoresis on 7–14% polyacrylamide gradient gels as described (15). Gels were treated with Enhance (New England Nuclear, Boston, MA) before exposure to Kodak XRP films (Eastman Kodak Co., Rochester, NY). The film was exposed using intensifying screens at −70°C for 48 h.

Preparation and Hybridization of Lung Cancer Cell Line DNAs and RNAs. Cells were grown as described above. DNA was prepared from each cell line and 15 μg was digested with *Eco*RI, electrophoresed on a 0.8% agarose gel, denatured, and transferred to nitrocellulose (16). Hybridization was performed using 50% dextran sulfate (17) with the ^{32}P-labeled probe and washing at 52°C (18). The HLA probe used was the human cDNA plasmid, psw, obtained from Dr. Sherman Weissman (19). The β_2m probe used was a human cDNA plasmid obtained from Dr. R. Bruce Wallace (20).

A postnuclear supernatent prepared from fresh cell cultures (21) was phenol/chloroform extracted and ethanol precipitated. 10 μg of total cytoplasmic RNA was denatured and electrophoresed on a 1% agarose-formaldehyde gel (22), that had been modified by using 0.22 M formaldehyde in the gel and electrophoresing at 160 V for 4 h. The gel was soaked in 3 M NaCl, 0.3 M sodium citrate. Transfer, hybridization, and washing were essentially as described for DNA.

Interferon Induction of HLA-frame and β_2m Proteins. Human recombinant leukocyte A interferon was supplied by Hoffmann-La Roche, Inc., Nutley, NJ. Its titer was 3×10^6 reference units per milliliter, and its specific activity was 6×10^5 reference units per milligram of protein. Human gamma interferon (Meloy Laboratories, Springfield, VA) had a titer of 1×10^6 reference units per milliliter and a specific activity of 1×10^6 reference units per milligram of protein. Seven SCLC lines, five NSCLC lines, and one B lymphoblastoid line were grown in microtiter dishes at 10^5 cells per well. Gamma interferon was added to triplicate wells of each line at concentrations of 0–10^4 U/ml. After 48 h, cells were washed three times in PBS, transferred to polyvinylchloride plates (Dynatech Laboratories, Inc., Alexandria, VA) and fixed with 0.25% glutaraldehyde. The plates were washed twice with PBS and blocked with 1% BSA. Each line was assayed at each interferon concentration with 10 μg/ml monoclonal antibodies W6/32, L-368, and (Becton Dickinson Monoclonal Antibody Center) HLA-DR. After 1 h incubation, plates were washed and incubated for 1 h with 1:1000 dilutions of affinity-purified rabbit anti–mouse IgG. After washing, plates were incubated with 40,000 cpm of ^{125}I–protein A for 1 h, washed, and read on a gamma counter.

Two SCLC lines (H-209, H-146) were also grown and assayed similarly with recombinant leukocyte A interferon, with assays performed at 24, 48, 72, and 96 h after exposure to the interferon. Larger wells, containing 10^6 cells per well of SCLC line NCI-H446, were assayed on an indirect immunofluorescence assay with either PBS or 10^4 U/ml of leukocyte A interferon at 24, 48, and 72 h after incubation. Indirect immunofluorescence assays were performed with W6/32 and L-368 as previously described.

Immunohistochemistry. Malignant human tissues and cultured cells were obtained, formalin-fixed, embedded, cut, and stained as described (23). Control and interferon-treated cells were cytocentrifuged onto glass slides and fixed for 10 min with acetone at 4°C. The slides were incubated at room temperature with either anti-HLA-A,B,C, anti-β_2m, anti-HLA-DR, or control antibodies, for 45 min. After rinsing the slides, the cells were exposed to biotin-labeled goat anti–mouse Ig for 30 min. The slides were incubated in methanol H_2O_2 solution for 30 min before the application of avidin-biotin-peroxidase complex (ABC), followed by diaminobenzidine–nickle chloride–H_2O_2 solution (24). The smears were counterstained with methyl green. All slides were reviewed by three clinical pathologists (A. Gazdar, I. Linnoila, and S. Martin).

Results

SCLC Cultured Cell Lines Express Greatly Reduced Amounts of Surface HLA or β_2m Antigens. Six independent monoclonal antibodies specific for invariant determinants on the class I HLA-A,B,C and β_2m proteins were tested for binding to SCLC, NSCLC, and non-lung cancer lines. In protein A–radiobinding assays, high levels of binding were seen with the anti-HLA antibodies to NSCLC and non-lung cancer lines and approached saturation at 10 μg/ml antibody per 10^5 target cells (Table I). In contrast, no or greatly reduced binding was noted with the small cell lines, depending on the antibody used. A single exception was line NCI-H526, an SCLC line by morphology, which consistently had high expression of HLA antigenic determinants, approaching the NSCLC levels, with the three monoclonal antibodies used. A similar pattern of results was noted with the

TABLE I

Binding of Monoclonal Antibodies Against HLA-A,B,C and β_2m Antigens to Human Cancer Lines in Solid Phase Radioimmunoassay

Cancer Type	Binding of monoclonal antibodies (cpm ^{125}I–protein A bound over background/10^5 cells) against:					
	HLA-A,B,C			β_2-Microglobulin		
	W6/32	9456-SA*	H-0592	BBM.1	L-368*	H-0602*
SCLC						
NCI-H69	0	133	394	0	0	1,216
NCI-H146	0	589	55	0	0	624
NCI-H209	0	362	153	0	0	484
NCI-H60	0	38	339	0	0	627
NCI-H128	0	339	0	0	0	4,368
NCI-H82	0	257	44	0	0	241
NCI-N417	0	244	182	0	0	381
NCI-H187	175	616	493	0	0	2,814
NCI-H446	0	622	255	0	364	797
NCI-H524	0	331	81	0	0	354
NCI-H526	1,624	9,668	14,388	5,214	17,663	36,819
NSCLC						
NCI-H23	1,558	13,437	19,317	3,450	18,934	36,762
NCI-H157	4,853	13,452	16,499	8,590	23,758	35,594
NCI-H125	1,399	13,513	18,699	3,000	22,207	33,672
NCI-H292	191	2,780	1,910	NT	NT	21,531
NCI-A427	NT	11,438	7,742	6,990	13,601	20,940
NCI-H226	NT	16,490	2,465	2,018	2,401	4,916
Other						
NCI-H234	1,190	14,357	17,624	7,248	24,005	30,786
NCI-H102	938	13,550	12,096	5,370	25,246	24,242

Assays were performed using a ^{125}I–protein A assay with commercial monoclonal antibodies at a concentration of 10 μg/ml. Results are the average of triplicate determinations (<20% variance between wells for any one test). The negative control was obtained by substituting a class-matched indifferent myeloma protein for the specific monoclonal antibody. Control radiobinding counts varied from 100 to 800 with the different antibodies. The numbers shown represent the cpm of the test well minus the cpm of the negative control. NT, not tested.

* Monoclonal antibody assays using a rabbit anti–mouse IgG connecting antibody.

monoclonal antibodies directed against β_2m, with a strong reaction to NSCLC noted with three different antibodies. The 11 SCLC lines tested, with the striking exception of NCI-H526, were negative with two of the anti-β_2m antibodies and slightly positive with the other antibody, compared with a control IgG mouse myeloma protein. The ratios of NSCLC-to-SCLC binding of these antibodies was up to 100:1 with both HLA,A,B,C and β_2m monoclonal antibodies.

13 SCLC and four NSCLC cell lines were tested in indirect immunofluorescence assays for HLA-A,B,C and β_2m expression, using live cells, to see if a subpopulation of SCLC expressed large amounts of the antigens. While >85% of the NSCLC cells were brightly fluorescent, SCLC cells showed only faintly positive cells. However, this positivity was present on the large majority of the cells. The median fluorescence of the SCLC cells was 10–100-fold less than the median fluorescence of the NSCLC cells with both HLA and β_2m monoclonal antibodies (data not shown). The only overlap in fluorescence intensity between SCLC and NSCLC with each antibody was again seen in line NCI-H526, which had much greater expression of HLA and β_2m antigens than any other SCLC line.

Fresh SCLC Tumor Specimens Do Not Express Surface HLA-A,B,C. To determine whether the loss of class I histocompatibility antigens occurred while the tumor was in the patient or only after cell culture, we used tumor samples taken directly from SCLC and NSCLC patients for immunohistochemical staining with 9456-SA, a monoclonal antibody reactive to HLA-A,B,C. The staining of three SCLC samples revealed that uncultured SCLC cells had markedly reduced or undetectable HLA-A,B,C expression (data not shown). Similarly, staining of SCLC cells adjacent to bronchus or skin with a heteroantisera reactive to β_2m showed that the surrounding nonmalignant cells were positively stained with the antisera, but that the SCLC cells were negative (Fig. 1).

SCLC Cells Synthesize Greatly Reduced Amounts of HLA-A,B,C and β_2m Proteins. To determine whether the lack of expression on the cell surface of HLA-A,B,C and β_2m resulted from a deficiency of peptide chain synthesis, we performed immunoprecipitation with antibodies directed against HLA-A,B,C and β_2m determinants. We used the monoclonal antibody W6/32 to precipitate the HLA-A,B,C antigen in 12 SCLC lines and five NSCLC lines (Fig. 2). The SCLC lines had a marked reduction in HLA-A,B,C protein immunoprecipitated compared with the NSCLC lines, with the exception again of NCI-H526. Densitometric analysis of the SCLC HLA protein bands showed that the median SCLC line synthesized 1/20th the amount of HLA protein compared with the median NSCLC line (Table II). In the NSCLC lines, a 12,000 mol wt band was also noted, presumably arising from co-immunoprecipitated β_2m bound to the HLA-A,B,C molecule. This was not seen in any of the SCLC lines. This co-immunoprecipitation of β_2m has been previously described with W6/32 (25).

To directly precipitate β_2m, we used an affinity-purified rabbit anti–human β_2m heteroantiserum (this serum behaved similarly to the monoclonal antibodies used in the radiobinding assays described above). Using the same 12 SCLC and five NSCLC lines as in the HLA immunoprecipitation, we found a marked reduction in the 12,000 mol wt β_2m bands precipitated in SCLC compared with NSCLC (Fig. 3). Densitometry analysis showed the median value of the SCLC

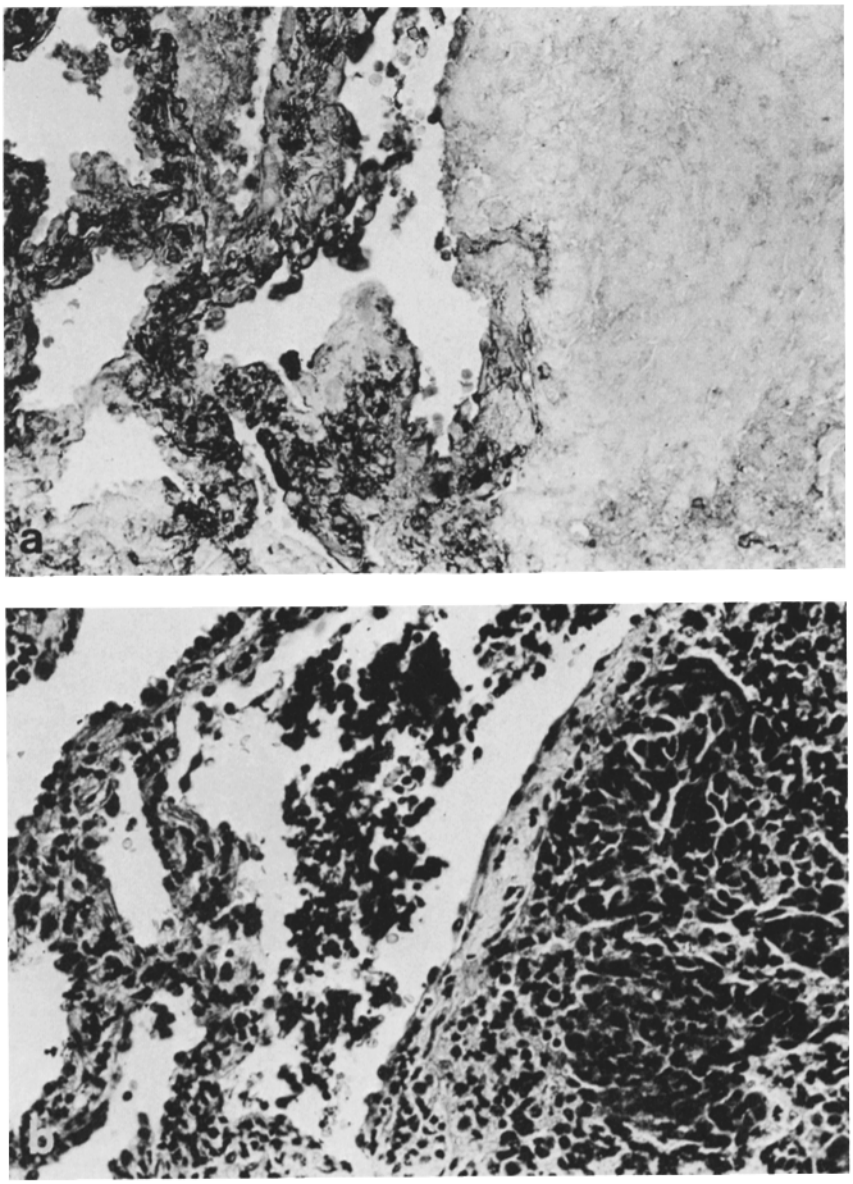

FIGURE 1. Photomicrographs of SCLC invading lung parenchyma in vivo. (*A*) After immunohistochemical staining with rabbit anti-β_2m, pulmonary alveolar cells (*left*) show marked immunoreactivity as opposed to the unstained tumor (*right*). (*B*) A serial section, 50 μm apart, stained with routine hematoxylin and eosin. The alveoli (*left*) contain cellular debris; SCLC is seen on the *right*.

β_2m bands to be 1/10th of the median NSCLC value (Table II). Of interest is the abnormally high molecular weight of the β_2m band seen in NCI-H446, a SCLC line (Fig. 3).

SCLC Cells Contain Markedly Reduced Levels of HLA-A,B,C and β_2m mRNAs. Knowing that the diminished cell surface expression of class I MHC

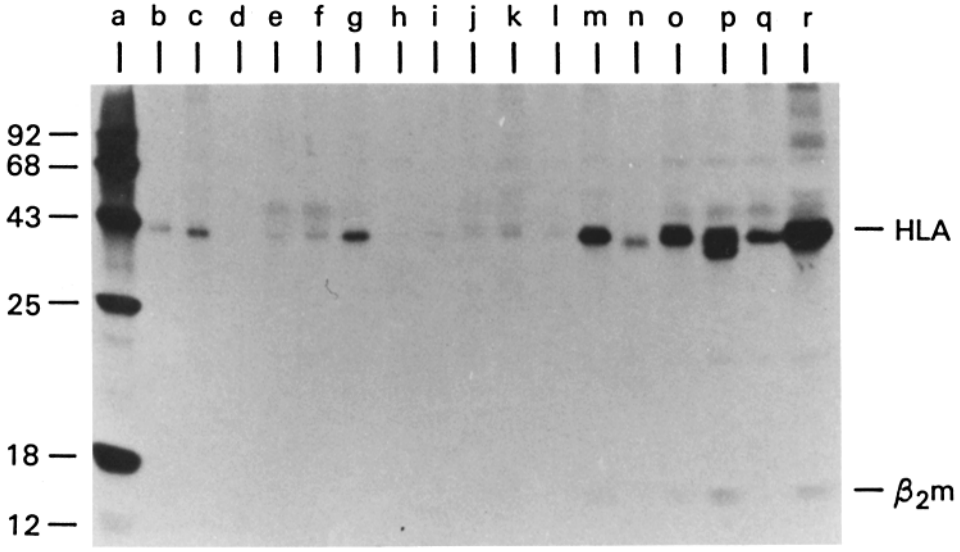

FIGURE 2. Immunoprecipitation analysis of HLA-A,B,C in lung cancer cell lines. [^{35}S]Methionine-labeled SCLC and NSCLC lysates were incubated with an anti-HLA-A,B,C monoclonal antibody in an immunoprecipitation assay. The HLA-A,B,C antigen band is at 45,000 mol wt, coprecipitated β_2m is at 12,000 mol wt. Lanes *b* through *m* are SCLC cell lysates immunoprecipitated with the antibody; *n* through *q* are NSCLC cell lysates. (*a*) Molecular weight standard; (*b*) NCI-H60; (*c*) NCI-H69; (*d*) NCI-H82; (*e*) NCI-H128; (*f*) NCI-H146; (*g*) NCI-H187; (*h*) NCI-H209; (*i*) NCI-H372; (*j*) NCI-H417; (*k*) NCI-H446; (*l*) NCI-H524; (*m*) NCI-H526; (*n*) NCI-H23; (*o*) NCI-H125; (*p*) NCI-H157; (*q*) NCI-A427; (*r*) NCI-H128 BL (B lymphoblastoid).

antigens was correlated with greatly reduced immunoprecipitable peptide chains, we next investigated class I MHC mRNA production. We analyzed RNA blots from 12 well-characterized human lung cancers with molecularly cloned HLA and β_2m cDNA probes. The HLA cDNA probe used (psw) corresponds to an HLA-B locus alloantigen (19), and detects a single RNA band of ~1,400 nucleotides. When NSCLC total cellular RNA was probed with psw, a single distinct band of 1.4 kilobase (kb) was seen (Fig. 4). Of the 10 SCLC lines tested, several failed to give a positive signal, and most gave a faint positive signal only after prolonged exposure of the gel. Densitometric analysis indicated that the median density of the SCLC HLA mRNA binds was less than one-fifth that of the NSCLC values (Table II). As a positive control, we used a B lymphoblastoid line (NCI-H128 BL) derived from the same patient as the NCI-H128 SCLC line; it showed a strong, single, 1.4 kg HLA mRNA band.

The β_2m cDNA probe used detects a single RNA band of 620 nucleotides (20). Using the same cell lines as in the psw assay, we found a 0.6 kb band in the B lymphoblastoid and lung adenocarcinoma lines (Fig. 5). The 10 SCLC lines tested showed positive but reduced signals with median densitometric β_2m mRNA levels less than one fifth of the NSCLC values (Table II). The SCLC line NCI-H526 was again the exception, with levels of HLA and β_2m mRNA comparable to the NSCLC levels, correlating with its increased HLA and β_2m protein synthesis.

TABLE II

Relative Amounts of HLA-A,B,C and β_2m Protein and mRNA Bands in SCLC and NSCLC

Cell line	Percent HLA RNA	Percent HLA protein	Percent β_2m RNA	Percent β_2m protein
SCLC				
NCI-H372	NT	1	8	10
NCI-H146	1	5	1	16
NCI-H209	NT	1	NT	8
NCI-H128	2	3	12	15
NCI-H60	NT	2	NT	9
NCI-H417	5	2	8	11
NCI-H187	7	9	8	24
NCI-H82	NT	1	NT	24
NCI-H249	9	NT	22	NT
NCI-H446	NT	4	NT	31
NCI-H69	10	6	18	11
NCI-H524	12	2	8	29
NCI-H231	15	NT	22	NT
NCI-H526	65	24	53	55
NSCLC				
NCI-H23	27	6	38	17
NCI-H125	66	34	58	131
NCI-H157	NT	47	NT	90
NCI-A427	NT	24	NT	20
B Lymphocyte				
NCI-H128 BL	100	100	100	100

Assays were performed using radioautograms developed on Kodak XRP film. Results compare the relative densities of HLA and β_2m RNA and protein bands in SCLC and NSCLC cell lines after standard immunoprecipitation and Northern blotting procedures. Relative densities were compared as percentages of the density of NCI-H128 BL, an Epstein-Barr virus–transformed B lymphoblastoid line derived from a SCLC patient. Densitometry measurements were performed on a DU-8 densitometer (Beckman Instruments, Inc., Fullerton, CA). NT, not tested.

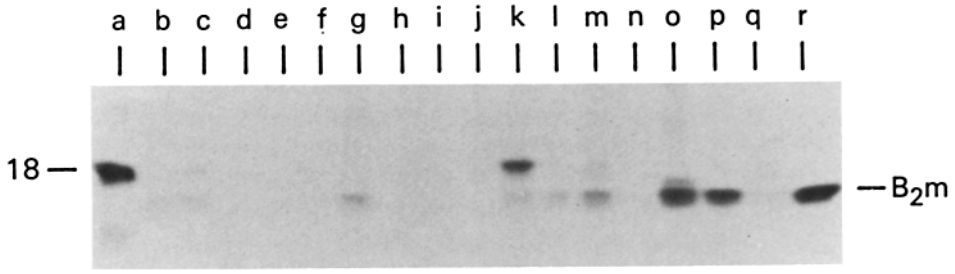

FIGURE 3. Immunoprecipitation analysis of β_2m in lung cancer cell lines. [^{35}S]Methionine-labeled SCLC and NSCLC lysates were incubated with an anti-β_2m heteroantisera in an immunoprecipitation assay. The β_2m antigen band is at 12,000 mol wt. Lanes *b* through *m* are SCLC cell lysates immunoprecipitated with the antisera; *n* through *q* are NSCLC cell lysates. (*a*) Molecular weight standard; (*b*) NCI-H60; (*c*) NCI-H69; (*d*) NCI-H82; (*e*) NCI-H128; (*f*) NCI-H146; (*g*) NCI-H187; (*h*) NCI-H209; (*i*) NCI-H372; (*j*) NCI-H417; (*k*) NCI-H446; (*l*) NCI-H524; (*m*) NCI-H526; (*n*) NCI-H23; (*o*) NCI-H125; (*p*) NCI-H157; (*q*) NCI-A427; (*r*) NCI-H128 BL (B lymphoblastoid).

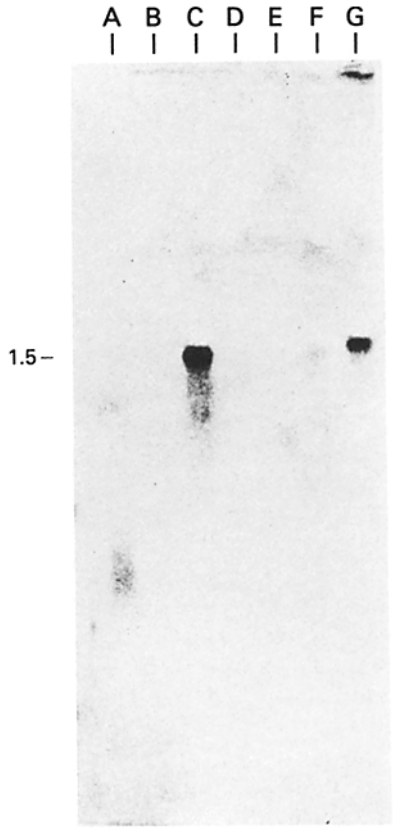

FIGURE 4. Northern blot analysis of RNA prepared from fresh cell cultures. 10 μg of total cytoplasmic RNA was denatured and electrophoresed on a 1% agarose-formaldehyde gel. The HLA probe was a human cDNA plasmid, psw, which hybridizes to a single 1.4 kb RNA band. Radioautography was performed with a 96 h exposure. (*A*) Lambda HindIII-digested genomic DNA; (*B*) NCI-N417 (SCLC-v); (*C*) NCI-H128 BL (immortalized lymphoblastoid line); (*D*) NCI-H128 (SCLC); (*E*) NCI-H146 (SCLC); (*F*) NCI-H69 (SCLC); (*G*) NCI-H23 (NSCLC).

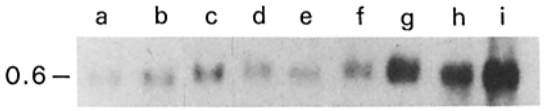

FIGURE 5. Northern blot analysis of RNA prepared from fresh cell cultures. 10 μg of total cytoplasmic RNA was denatured and electrophoresed on a 1% agarose-formaldehyde gel. The β_2m probe was a human cDNA fragment that hybridizes to a single 0.6 kb RNA band. Radioautography was performed with a 96 h exposure. (*a*) NCI-H187 (SCLC); (*b*) NCI-H231 (SCLC); (*c*) NCI-H524 (SCLC-v); (*d*) NCI-N417 (SCLC-v); (*e*) NCI-H69 (SCLC); (*f*) NCI-H249 (SCLC); (*g*) NCI-H23 (NSCLC); (*h*) NCI-H125 (NSCLC); (*i*) NCI-H128 BL (B lymphoblastoid).

HLA and β_2m Genes Are Present in SCLC. Because of decreased mRNA production we next asked if deletion or rearrangement of class I antigen genes had taken place. We analyzed EcoRI-digested genomic DNA from eight well-characterized human lung cancer cell lines with the HLA and β_2m cDNA probes, using the Southern nitrocellulose transfer method (16). The polymorphic band-

ing pattern detected by the psw probe in the SCLC lines was similar to the signals detected with the NSCLC and control cell line lanes (data not shown). The DNA banding pattern detected with the β_2m probe was identical in the SCLC and NSCLC lines (data not shown).

Interferon Induces Increased Expression of Surface HLA-A,B,C and β_2m Antigens in SCLC Cells. Interferon has been shown to increase HLA and β_2m expression in several systems (26, 27). SCLC cells were incubated with interferon and the class I MHC antigen expression was monitored. Recombinant leukocyte A interferon increased the expression of HLA-A,B,C and β_2m surface antigens in radiobinding (Fig. 6) and indirect immunofluorescence assays (Fig. 7). Gamma interferon also increased β_2m expression 3–50-fold and increased HLA-A,B,C antigen 2–24-fold (Table III). In contrast, interferon had no effect on HLA-DR expression, which remained low (data not shown). The expression of both antigens increased with increasing doses up to 10^4 U/ml of both gamma and recombinant leukocyte A interferons. The effect of both gamma and leukocyte A interferons in all assays was complete at 24 h and was not appreciably altered between 24 and 96 h. In addition both interferons also increased HLA-A,B,C and β_2m expression on the several NSCLC lines and the B lymphoblastoid line tested. In indirect immunofluorescence assays with SCLC line NCI-H446, the number of antigen-positive cells increased; also, fluorescence increased >10-fold in both HLA-A,B,C and β_2m assays after incubation with 10^4 U/ml of recombinant leukocyte A interferon, compared with treatment with the PBS control (Fig. 7).

The SCLC line NCI-H69 was used for immunohistochemical staining with an anti-HLA monoclonal antibody before and after interferon treatment (data not shown). The anti-HLA antibody was minimally reactive to NCI-H69 cells without

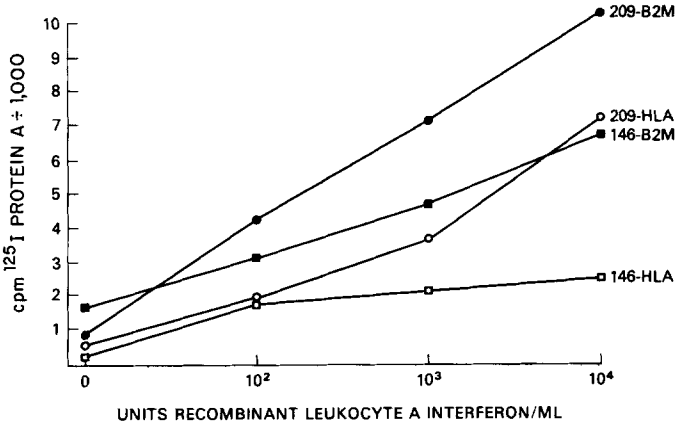

FIGURE 6. Radiobinding assay using W6/32, a monoclonal antibody reactive to HLA-A,B,C, and L-368, a monoclonal antibody reactive to β_2m, to determine expression of these antigens on two SCLC lines (NCI-H209 and NCI-H146) after incubation with varying concentrations of recombinant leukocyte A interferon. Interferon was added in triplicate to wells of each line at concentrations of 0–10^4 U/ml. After 24 h, cells were fixed with glutaraldehyde and assayed with the monoclonal antibodies. Plates were sequentially incubated with an affinity-purified rabbit anti-mouse IgG antiserum and ^{125}I-labeled protein A, and the wells were counted on a gamma counter.

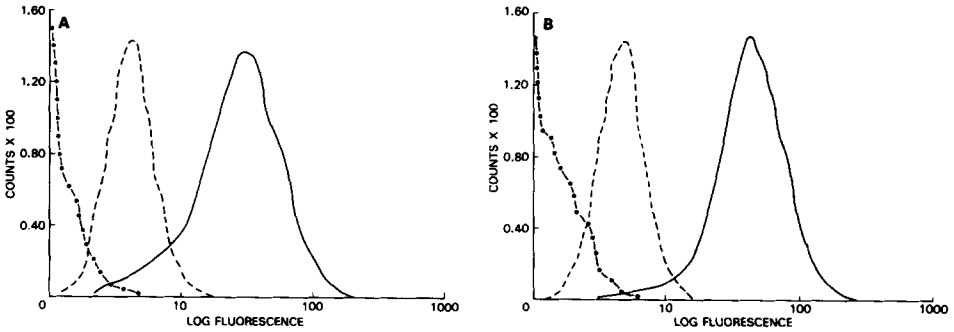

FIGURE 7. Positive HLA-A,B,C and β_2m fluorescence in SCLC line NCI-H446 after interferon incubation. Cells were incubated with either PBS or 10^4 U/ml of recombinant leukocyte A interferon. After 24 h, the live cell suspensions were incubated with a monoclonal antibody to HLA-A,B,C (*A*) or to β_2m determinants (*B*). Cells were then incubated with a fluuoresceinated goat anti-mouse secondary antibody. Fluorescence was analyzed in a Coulter Epics V cell sorter. The NSCLC line NCI-H157 was used, without interferon pretreatment as a positive control in each case.

TABLE III

Induction of HLA-A,B,C and β_2m Antigens by Interferon in SCLC Cell Lines

Cell line	Fold induction of:	
	HLA-A,B,C	β_2m
NCI-H60	4	17
NCI-H69	10	51
NCI-H128	18	34
NCI-H146	24	5
NCI-H187	2	3
NCI-H209	14	12
NCI-H417	3	23

Assays were performed on SCLC cells incubated at 10^5 cells per well in microtiter wells with 10^4 U/ml of gamma interferon for 48 h. Control wells with no interferon were prepared for each cell line. After 48 h, cells were washed with PBS and fixed to polyvinylchloride plates with 0.25% glutaraldehyde. The plates were reacted with 50 μl of W6/32 (reactive with HLA-A,B,C) or L-368 (reactive with β_2m). Both antibodies were at a concentration of 10 μg/ml. The plates were washed, then serially incubated with affinity-purified rabbit anti-mouse IgG and ^{125}I-labeled staphylococcal protein A. Counts were read on a gamma counter. Results represent the mean number of counts for each cell line after interferon incubation divided by the mean number of counts of the control wells.

interferon treatment but became markedly positive after 48 h exposure to 10^4 U/ml of gamma interferon incubation. In contrast, control immunoperoxidase staining with a monoclonal antibody reactive to HLA-DR remained negative after interferon incubation (data not shown).

Discussion

We have demonstrated that SCLC cells express greatly reduced amounts of class I cell surface antigens, their immunoprecipitable proteins, and their corre-

sponding mRNAs, but have apparently intact class I MHC genes, indicating a block of mRNA transcription. In this respect, SCLC cells clearly differ from NSCLC cells in class I MHC antigens. Both SCLC and NSCLC have low expression of class II HLA-DR antigens, as demonstrated by protein A radiobinding and indirect immunofluorescence assays (data not shown).

Including this report, three classes of human carcinomas have been shown to have frequent deficits of class I MHC antigen expression: germ cell cancers, neuroblastoma, and now, small-cell lung cancer. The recently described HLA-A,B,C deficiencies in neuroblastoma are particularly interesting since they extend the biochemical similarities noted between adrenergic neuroblastomas and SCLC. Both of these tumors produce L-dopa decarboxylase (8), have similar patterns of reactivity to monoclonal antibodies developed in our laboratory (11, 29), and have similar cell surface protein phenotypes (30). The markedly reduced surface expression of class I MHC peptides in both cell types raises the question of whether other normal and malignant APUD cells lack HLA-A,B,C and β_2m expression. However, neuroblastoma cell lines produce β_2m while displaying no detectable HLA-A,B,C antigen (2), whereas SCLC lines appear to have deficiencies of both HLA framework and β_2m proteins (though the β_2m deficiency may be less marked than the HLA-A,B,C loss). There is a reproducible spectrum in the degree of HLA-A,B,C deficiency in the SCLC lines with a roughly parallel decrease in internal β_2m peptide. While small amounts of β_2m proteins are translated in all SCLC cell lines, the absence of intracellular HLA-A,B,C protein may prevent β_2m from getting to the cell surface, explaining the more striking deficiency of β_2m protein by radiobinding assays. Alternatively, an altered form of internal β_2m peptide, as seen in NCI-H446, may not be transported to the cell surface. SCLC is unique among human tumor lines in having deficiencies of expression of both class I chains: the germ cell tumors and occasional breast, colon, and lymphoma lines with class I MHC deficiencies all appear to lack only one of the two antigens. In the Daudi Burkitt's lymphoma line (31), the inability to produce β_2m may be associated with major deletions on chromosome 15, where the β_2m gene is located (31). Karyotyping of multiple SCLC lines (10) has not shown any obvious abnormalities of chromosomes 15 or 6, where the human class I MHC genes are located.

The closest parallel to the loss of both HLA-A,B,C and β_2m antigens in SCLC lines appears to be the F9 mouse teratocarcinoma line. This line has been shown to have an RNA transcription block of both class I MHC antigens (6). Of interest, F9 cells have been shown to have a surface glycoprotein identified as the stage-specific embryonic antigen (SSEA) (32, 33). SSEA is found in the early embryo and disappears at the same developmental stage that H-2 MHC antigens appear (34). The majority of SCLC lines have a dominant carbohydrate antigen lacto-*N*-fucopentose III (LNFP3), which is identical to the determinant recognized by monoclonal antibodies made against SSEA (35). However, using [^{35}S]methionine-labeled SCLC cells, we have so far been unable to immunoprecipitate a protein with 534-F8, a monoclonal anti-SCLC antibody reactive to LNFP3 (35). The F9 teratocarcinoma line has been shown to differentiate with retinoic acid treatment (36), and differentiated clones were found to express H-2 and β_2m antigens but to have lost SSEA expression (36). In contrast, treatment of SCLC cells with recombinant leukocyte A interferon did not cause a reduction in their high

reactivity to 534-F8 after induction of HLA-A,B,C and β_2m peptide synthesis (data not shown).

Interferons have been found to augment expression of class I MHC antigen expression in melanoma cells (26), and have been shown to induce mRNA for MHC antigens in human fibroblasts and lymphoblastoid cells (27). The current experiments, however, represent the first reported example of interferons increasing the RNA transcription of class I MHC antigens that had been markedly diminished. There is a presumed role for interferon in increasing HLA expression, as part of the immune response of a virally infected host, for the elimination of HLA-matched infected cells. Increased HLA expression in SCLC might be expected to facilitate the destruction of cells bearing tumor-associated antigens.

With 25,000 cases of SCLC diagnosed in the United States every year, SCLC is the most common human malignancy associated with major deficiencies of class I antigens. As a tumor that can be grown in cell culture with high efficiency it is a valuable model for studies on human histocompatibility antigen expression. Our results also raise a basic clinical question. A growing body of literature (37–39) suggests that class II MHC antigens participate in the lysis of tumor cells by cytotoxic T lymphocytes. It has been suggested (40) that a lack of HLA-A,B,C may offer a growth advantage to tumors with weak expression of the molecules. Studies with melanoma line variants that were low in class I MHC expression (41) showed that these variants were more likely to metastasize from locally implanted mouse tumors than were lines high in MHC expression. In this context we note that SCLC is clinically characterized by aggressive growth and early metastasis. Since NSCLC (which requires different treatment than SCLC) expresses both HLA-A,B,C and β_2m antigens, these markers are of immediate diagnostic value in tumor typing. Administering interferon to SCLC patients after their tumor bulk had been reduced by chemotherapy and radiotherapy could be of value in delaying or preventing tumor recurrence.

The mechanism of decreased class I MHC mRNA transcription in SCLC cells is unknown, but may be comparable to the decrease in histocompability antigen transcription seen in rat cells after malignant transformation by the E1a region of adenovirus strain 12 (7). The small-cell variant lines have been shown to have an amplification of the c-*myc* oncogene (12) which, like E1a, appears to have an establishment function in cell transformation (42). Cell lines of neuroblastoma, the other non–germ cell tumor with consistent decreases in class I MHC antigen expression, also have amplification of a c-*myc*-related oncogene, N-*myc* (43). In general, the SCLC lines with the greatest amplification of c-*myc* also had the lowest measurable levels of class I histocompatibility antigens. Of interest, NCI-H23, a lung adenocarcinoma line amplified for c-*myc*, had significantly lower amounts of HLA-A,B,C protein and mRNA than the other NSCLC lines tested. Decreased transcription of class I MHC message may be a common aspect of malignant transformation with the functional class of oncogenes that include, c-*myc*, N-*myc*, and E1a.

Summary

We have found markedly deficient expression of the class I major histocompatibility antigens HLA-A,B,C and β_2m on human small-cell lung cancer (SCLC) lines and fresh tumor samples. The deficit of HLA-A,B,C and β_2-microglobulin

(β_2m) antigen expression was demonstrated with both radiobinding assays and indirect immunofluorescence assays. Immunoprecipitation of metabolically labeled cells with antibodies to class I antigens showed most SCLC lines to have synthesized almost no β_2m and HLA-A,B,C proteins. Northern blot analysis, using human HLA-A,B, and β_2m cDNA probes, showed that almost all SCLC lines tested had markedly decreased amounts of HLA and β_2m mRNA, but both gene products could be induced with interferon treatment of SCLC lines. We conclude that human SCLC, in contrast to other lung cancer types, is characterized by greatly reduced transcription of HLA-A,B,C and β_2m genes, which suggests the existence of a mechanism for evading the host immune response to the tumor and of an E1a-like product in this type of tumor cell.

We thank S. Stephenson, J. Fedorko, P. Jewitt, J. Heard, and H. Sims for technical help and for providing tissue culture reagents; Drs. M. Kuehl, R. Currie, J. Battey, P. Reynolds, and D. Margulies for advice or for reviewing the manuscript. We thank Dr. S. Weissman for the gift of the cloned HLA cDNA probe and Dr. B. Wallace for the gift of the cloned β_2m cDNA probe. We also thank Hoffman-La Roche Co. for the gift of recombinant leukocyte A interferon.

Received for publication 18 July 1984 and in revised form 23 January 1985.

References

1. Faulk, W. P., A. R. Sanderson, and A. Temple. 1977. Distribution of MHC antigens in human placental chorionic villi. *Transplant. Proc.* 9:1379.
2. Lampson, L. A., C. A. Fisher, and J. P. Whelan. 1983. Striking paucity of HLA-A,B,C and β_2-microglobulin on human neuroblastoma cell lines. *J. Immunol.* 130:2471.
3. Trowsdale, J., P. Travers, W. F. Bodmer, and R. A. Patillo. 1980. Expression of HLA-A, -B, and -C and β_2-microglobulin antigens in human choriocarcinoma cell lines. *J. Exp. Med.* 152(Suppl.):11s.
4. Arce-Gomez, B., E. A. Jones, C. J. Barnstable, E. Solomon, and W. F. Bodmer. 1977. The genetic control of HLA-A and B antigens in somatic cell hybrids: requirements for β_2-microglobulin. *Tissue Antigens.* 11:96.
5. Travers, P. J., J. L. Arklie, J. Trowsdale, R. A. Patillo, and W. F. Bodmer. 1982. Lack of expression of HLA-ABC antigens in choriocarcinoma and other human tumor cell lines. *Natl. Cancer Inst. Monogr.* 60:175.
6. Croce, C. M., A. Linnenbach, K. Huebner, P. R. Parnes, D. M. Margulies, E. Appella, and J. G. Seidman. 1981. Control of expression of histocompatibility antigens (H-2) and β_2-microglobulin in F9 teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA.* 78:5754.
7. Schrier, P. I., R. Bernards, R. T. M. J. Vaessen, A. Houweling, and A. J. van der Erb. 1983. Expression of class I major histocompatibility antigens switched off by highly oncogenic adenovirus 12 in transformed rat cells. *Nature (Lond.).* 305:771.
8. Gazdar, A. F., D. N. Carney, E. K. Russell, H. Simms, S. B. Baylin, P. A. Bunn, and J. N. Minna. 1980. Small-cell carcinoma of the lung: establishment of continuous clonable cultures having amine precursor uptake and decarboxylation properties. *Cancer Res.* 40:3502.
9. Carney, D. N., P. A. Bunn, A. F. Gazdar, and J. D. Minna. 1981. In vitro cloning of

small-cell carcinoma of the lung. *In* Small Cell Lung Cancer. A. Greco, P. A. Bunn, R. Oldham, editors. Grune & Stratton, Inc., New York.

10. Whang-Peng, J., P. A. Bunn, C. S. Kao-Shan, E. C. Lee, D. N. Carney, A. Gazdar, and J. D. Minna. 1982. A nonrandom chromosomal abnormality, del 3p (14–23), in human small-cell lung cancer (SCLC). *Cancer Genet. Cytogenet.* 6:119.
11. Cuttitta, F., S. Rosen, A. F. Gazdar, and J. D. Minna. 1981. Monoclonal antibodies which demonstrate specificity for several types of human lung cancer. *Proc. Natl. Acad. Sci. USA.* 78:4591.
12. Little, C. D., M. M. Nau, D. N. Carney, A. F. Gazdar, and J. D. Minna. 1983. Amplification and expression of the c-myc oncogene in human lung cancer cell lines. *Nature (Lond.).* 306:194.
13. Barnstable, C. J., W. F. Bodmer, G. Brown, G. Galfre, C. Milstein, A. F. Williams, and A. Ziegler. 1978. Production of monoclonal antibodies to group A erythrocytes, HLA and other human cell surface antigens: new tools for genetic analysis. *Cell.* 14:9.
14. Lampson, L. A. 1980. Immunoprecipitation with monoclonal antibodies. *In* Monoclonal Antibodies. R. Kennett, K. Bechtol, and T. McKearn, editors. Plenum Publishing Corp., New York. 395–397.
15. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
16. Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503.
17. Wahl, G. M., M. Stern, and G. R. Stark. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl paper and rapid hybridization by using dextran sulfate. *Proc. Natl. Acad. Sci. USA.* 76:3683.
18. Taub, R., I. Kirsch, C. Morton, G. Lenior, D. Swan, S. Tronick, S. Aaronson, and P. Leder. 1982. Translocation of the c-myc gene into the immunoglobulin heavy chain locus in human Burkitt lymphoma and murine plasmacytoma cells. *Proc. Natl. Acad. Sci. USA.* 79:7837.
19. Sood, A. K., D. Pereira, and S. M. Weissman. 1981. Isolation and partial nucleotide sequence of a cDNA clone for human histocompatibility antigen HLA-B by use of an oligonucleotide primer. *Proc. Natl. Acad. Sci. USA.* 78:616.
20. Suggs, S. V., R. B. Wallace, T. Hirose, E. H. Kawashima, and K. Itakawa. 1981. Use of synthetic oligonucleotides as hybridization probes: isolation of cloned cDNA sequences for human β_2-microglobulin. *Proc. Natl. Acad. Sci. USA.* 78:6613.
21. Gorman, C. M., G. T. Merlino, M. C. Willingham, I. Pastan, and B. H. Howard. 1982. The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of cells by DNA-mediated transfer. *Proc. Natl. Acad. Sci. USA.* 79:6777.
22. Lehrach, H., D. Diamond, J. M. Wozney, and H. Boedtker. 1977. RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. *Biochemistry.* 16:4743.
23. Hsu, S., and E. Soban. 1982. Color modification of DAB precipitation by metallic ions and its application for double immunohistochemistry. *J. Histochem. Cytochem.* 30:1079.
24. Hsu, S., L. Raine, and H. Fanger. 1981. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques. *J. Histochem. Cytochem.* 29:577.
25. Quarante, V., L. E. Walker, G. Ruberto, M. A. Pellegrino, and S. Ferrone. 1981. The free and the β_2-microglobulin-associated heavy chains of HLA-A,B alloantigens

share the same determinant recognized by the monoclonal antibody Q1/28. *Immunogenetics.* 13:285.

26. Basham, T. Y., M. F. Bourgeade, A. A. Creasey, and T. C. Merigan. 1982. Interferon increases HLA synthesis in melanoma cells: interferon-resistance and -sensitive cell lines. *Proc. Natl. Acad. Sci. USA.* 79:3265.
27. Fellous, M., U. Nir, D. Walloch, G. Merlin, M. Rubenstein, and M. Revel. 1982. Interferon-dependent induction of mRNA for the major histocompatibility antigens in human fibroblasts and lymphoblastoid cells. *Proc. Natl. Acad. Sci. USA.* 79:3082.
28. Tapia, F. J., J. M. Polak, A. J. A. Barbosa, P. J. Marengos, S. R. Bloom, C. Dermody, and A. G. E. Pearse. 1981. Neuron-specific enolase is produced by neuroendocrine tumors. *Lancet.* 1:241.
29. Rosen, S. T., J. L. Mulshine, F. Cuttitta, J. Fedorko, D. N. Carney, A. F. Gazdar, and J. D. Minna. 1984. Analysis of human small-cell lung cancer differential antigens using a panel of rat monoclonal antibodies: a common link between small-cell lung cancer, endodermal tumors and neuroblastoma. *Cancer Research.* 44:2052.
30. Baylin, S. B., A. F. Gazdar, J. D. Minna, and J. H. Shaper. 1982. Cell surface protein phenotype of human lung cancer in culture. Identification of common and distinguishing cell surface proteins on the membrane of different human lung cancer cell types. *Proc. Natl. Acad. Sci. USA.* 79:4650.
31. Goodfellow, P. N., E. A. Jones, V. Van Heyninger, E. Solomon, M. Bobrow, V. Miggiano, and W. F. Bodmer. 1975. The beta 2-microglobulin gene is on chromosome 15 and not in the HLA-A region. *Nature (Lond.).* 254:267.
32. Forman, J., and E. S. Vitetta. 1975. Absence of H-2 antigens capable of reacting with cytotoxic T cells on a teratoma line expressing a T/t locus antigen. *Proc. Natl. Acad. Sci. USA.* 72:3661.
33. Bennett, D. 1981. T/t locus, its role in embryogenesis and its relation to classical histocompatibility systems. *Prog. Allergy.* 29:35.
34. Solter, D., and B. B. Knowles. 1978. Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). *Proc. Natl. Acad. Sci. USA.* 75:5565.
35. Huang, L. C., M. Brockhaus, J. L. Magnani, F. Cuttitta, S. Rosen, J. D. Minna, and V. Ginsberg. 1983. Many monoclonal antibodies with an apparent specificity for certain lung cancers are directed against a sugar sequence found in lacto-*N*-fucopentose III. *Arch. Biochem. Biophys.* 220:318.
36. Knowles, B. B., S. Pan, D. Solter, A. Linnenbach, C. Croce, and K. Huebner. 1980. Expression of H-2, laminin and SV4OT and TASA on differentiation of transformed murine teratocarcinoma cells. *Nature (Lond.).* 288:615.
37. Schrader, J. W., and G. M. Edelman. 1976. Participation of the H-2 antigens of tumor cells in their lysis by syngeneic T cells. *J. Exp. Med.* 143:601.
38. Poupon, M. F., B. Payelle, and G. Lespinats. 1981. Adoption of tumor immunity: role of the major histocompatibility complex. *J. Immunol.* 126:2342.
39. Stukart, M. J., A. Vos, J. Boes, R. W. Melvold, D. W. Bailey, and C. J. M. Melief. 1982. A crucial role of the H-2 locus in the regulation of both the D- and the K-associated cytotoxic T lymphocyte response against Moloney leukemia virus, demonstrated with two D^b mutants. *J. Immunol.* 128:1360.
40. Bodmer, W. F. 1981. HLA structure and function: a contemporary view. *Tissue Antigens.* 17:9.
41. Baniyash, M., N. I. Smorodinsky, M. Yaakubovicz, and I. P. Witz. 1982. Serologically detectable MHC and tumor-associated antigens on B16 melanoma variants and humoral immunity in mice bearing these tumors. *J. Immunol.* 129:1318.

42. Land, H., L. F. Parada and R. A. Weinberg. 1983. Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. *Nature* (*Lond.*). 304:596.
43. Kohl, N. E., N. Kanda, R. R. Schreck, G. Bruns, S. A. Latt, F. Gilbert, and F. W. Alt. 1983. Transposition and amplification of oncogene-related sequences in human neuroblastomas. *Cell.* 35:359.