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Immunological Characterization of Cell Lines Established from Malignant and Normal Human Urothelium*

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Abstract—*Cell lines from normal or malignant human urothelium, described elsewhere by morphological, cell kinetic and genotypical criteria, have been characterized further by their response to antibody or lymphocyte mediated cytotoxicity* in vitro. *Four groups of cell lines were included:* (1) *two fast proliferating cell lines from normal urothelium,* (2) *three fast proliferating cell lines from transitional cell carcinoma (TCC) together with two transformed sublines of normal derived cell lines,* (3) *six slowly proliferating TCC lines, and* (4) *a line from squamous cell carcinoma. HLA antigen expression was demonstrated in the cell lines of groups* 1 *and* 3, *but not in lines from group* 2 *or* 4. *The sensitivity to spontaneous lymphocyte mediated cytotoxicity (SLMC) of cells in group* 1 *and* 2 *exceeded that of cells from group* 3 *by a factor of* 8. *The TCC line, HU* 456, *was more susceptible to SLMC than* T 24. *Specifically increased cytotoxicity of lymphocytes from patients suffering from interstitial cystitis (IC) against HU* 609 *from normal urothelium indicated that this line expresses tissue specific antigens, and at the same time that an immune reaction may be part of the pathogenesis of IC. By a sensitive test for antibody-dependent, cell-mediated cytotoxicity (ADCC) antibody activity against HU* 456 *was found in only one of nine TCC patients and none of five clinical controls. A pronounced non-disease-related blocking serum activity, frequently found, may account for some of the negative findings. The SLMC against HU* 456 *could be inhibited but not abolished by Fab fragments of rabbit anti-human immunoglobulin, indicating an ADCC mechanism as part of the SLMC.*

INTRODUCTION

CELL lines from normal or malignant human urothelium, established in our laboratory, and used as target cells in different immune investigations [1–3] together with the lines T 24 and HCV 29, have been allocated to four different subgroups [4, 5]: (1) fast proliferating cell lines from normal urothelium: HU 609 and HCV 29; (2) fast proliferating transitional cell carcinoma (TCC) cell lines and the transformed sublines of HU 609 and HCV 29: HU 456, HU 549, T 24, HU 609 T and HCV 29 T; (3) slowly proliferating TCC lines: HU 961, HU 1125, HU 1207, HU 1210, HU 1244 and HU 1264; and (4) one cell line from squanous cell carcinoma of the urinary bladder: HU 1305.

Several TCC cell lines, summarized elsewhere [4], have been used in investigation of lymphocyte-mediated cytotoxicity [6–13] or in tests for TCC-directed antibodies [6, 14, 15].

Since the immune reactions observed with different target cell lines may vary due to differences between the lines, and since these variations may be responsible for discrepancies among the findings of different investigations, a detailed immunological characterization of the lines used in our experiments has been considered essential.

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MATERIALS AND METHODS

Cell culture

Cell lines were kept in disposable tissue culture bottles (Nunc, Denmark) in FIB 41 B, which is a modified Eagles Minimal Essential Medium [16].

Lymphocyte preparation

Lymphocytes were prepared by defibrination, gelatin sedimentation, acryl wool incubation, and ammonium chloride lysis [1, 16].

HLA typing of cell lines

Nine HLA semi-specific antisera were kindly selected and provided in code by the Tissue Type Laboratory of Rigshospitalet. The Copenhagen University Hospital. An assay for complement-dependent cytotoxicity was carried out in microtiter plates, each containing 60 wells (Falcon 3034, or the corresponding from Nunc, Denmark). Each cell line was precultured in a microtiter plate for 72 hr in medium with 10% heat inactivated fetal bovine serum (FBS) at 5% CO_2 and 37°C and subsequently processed as follows. After removal of the medium phosphate buffered saline (PBS), pH = 7.4, was added to 12 wells in a plate, and each of 8 different antisera diluted 1:5 with PBS was added to groups of 6 wells in the same plate, 4 µl per well. The plates were then incubated at 5% CO_2 at 37°C for 1.5 hr, after which 4 µl rabbit nonimmune serum was added as a source of complement to all 60 wells in the plate. After a further 90 min incubation at 5% CO_2 and 37°C the cells were washed, fixed, stained, and counted semiautomatically as described elsewhere [16]. The percentage of cytotoxicity was estimated by comparing the mean of cell numbers in 6 wells incubated with a given HLA specific antiserum with the mean of cell numbers in 12 wells from the same plate incubated without HLA specific antiserum. An additional experiment was performed with the cell lines HU 1125 and HU 961, including only two antisera (Fig. 1).

Microcytotoxicity assay (MA)

Titration of cell mediated cytotoxicity was as previously described [1, 16].

ADCC

HU 456 cells were cultured in microtissue culture plates. Each experiment included the following groups of wells: (a) 12 wells incubated with medium alone; (b) 12 wells with effector lymphocytes (0.5×10^6 ml) from one healthy donor; and (c) groups of 6 wells containing lymphocytes at the same concentration as in (b) plus antiserum at a given concentration from a serial dilution. The plates were incubated for 44 hr, after which the remaining cells were counted as previously described [16]. The calculations of cytotoxicity were based on mean values of the remaining cells ($\bar{N}$) in the groups described. The background SLMC was calculated as $\frac{\bar{N}a - \bar{N}b}{\bar{N}a} \times 100\%$, and the ADCC as: $\frac{\bar{N}b - \bar{N}c}{\bar{N}b} \times 100\%$.

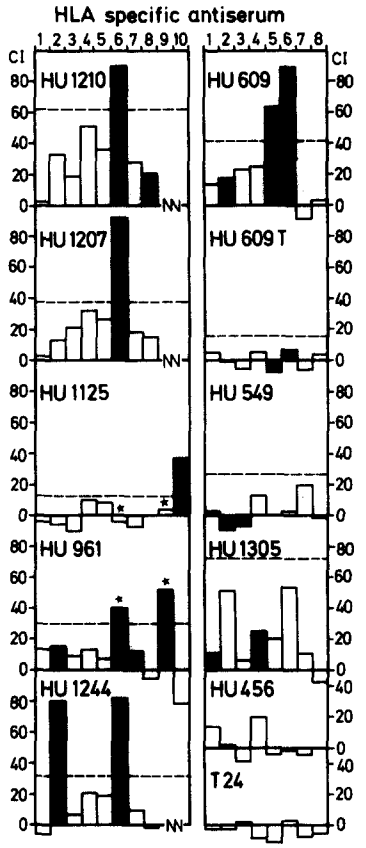

Fig. 1. Demonstration of HLA antigen in slowly proliferating TCC lines and line HU 609 from normal urothelium. Lack of HLA expression on transformed cells from HU 609 and fast proliferating cell lines from TCC or squamous cell carcinoma of the urinary bladder. Black columns indicate HLA specificities demonstrated on leucocytes of the cell line donor. HLA specificity of antisera: 1 = A1, 2 = B7, 3 = B8, 4 = B4a2, 5 = B5, 6 = A2, 7 = A1o + 25, 8 = A3, 9 = 6(A 2), 10 = A11. *The cell line number appears in the upper left corner of each histogram. CI = cytotoxicity index.*

$$CI = \frac{\text{remaining cells with complement} - \text{remaining cells with complement + antiserum}}{\text{remaining cells with complement}} \times 100\%.$$

** = duplicate experiments with the same antiserum in lines HU 1125 and HU 961. ---- = $\bar{X} + 2s$ of CI's with nonrelated antisera (open columns), calculated for each cell line.*

Inhibition of cytotoxicity by Fab fragments

Fab fragments of rabbit antihuman immunoglobulin were kindly prepared and provided by M. Troye [17]. Microcytotoxicity assay was carried out with Hu 456 as target cells as described [16]. A dilution of 1.25×10^6 lymphocytes/ml was applied to all wells in a plate except for 12 wells with medium alone. Another 6 wells were incubated with lymphocytes alone. The remaining wells were divided into 7 groups each containing 6 wells to which serial dilutions of a Fab solution were added.

RESULTS

HLA typing

The susceptibility of the cell lines to complement dependent lysis by 9 HLA specific antisera is demonstrated in Fig. 1, in which black columns represent target cell/antiserum combinations where a high degree of cytotoxicity was expected from the HLA typing of the respective donor patients. In the group of slowly proliferating cells (Fig. 1, left side) a strong cytotoxicity was seen in most cases when the antiserum corresponded to the HLA type of the tissue donor, whereas reactivities with antisera not directed at the appropriate type were low. When all reactivities found within this group of slowly proliferating cells were allocated to one of two groups, one with values expected to be high, and one with values expected to be low, the degree of cytotoxicity in the first group significantly exceeded that of the latter, $2P<0.02$ (Mann–Whitney's rank sum test). When $\bar{x}+2s$ of CI values with nonrelated antisera for each of the cell lines was used as the lower limit of positive specific reactions (indicated by horizontal dotted lines in Fig. 1), six of ten HLA antigens expected to be present on the slowly proliferating cell lines were demonstrated, but positive cytotoxicity with nonrelated antisera was not observed. The correlation between positive cytotoxicity in this test and in the routine HLA analysis of cell donor leucocytes was statistically significant, $2P<0.002$ (Fischer's exact test). Thus, HLA antigens could be demonstrated in all five cells lines.

Likewise, two of three HLA antigens supposed to be present on HU 609 could be demonstrated while no HLA antigens were present in this line after transformation or in the fast proliferating cell lines from TCC or squamous cell carcinoma of the bladder. The HLA types of donors of HU 456 and T 24 were not known.

Microcytotoxicity assay (MA)

Cytotoxicity was demonstrated both with slowly proliferating and with fast proliferating target cell lines. The lymphocyte concentration required for 50% cytotoxicity in slowly proliferating cell lines exceeded that required for a similar degree of cytotoxicity in fast proliferating cell lines by a factor of eight.

Figure 2 demonstrates the distributions of lymphocyte mediated cytotoxicity as measured by the integrated cytotoxicity index (ICI), tumor-specific cytotoxicity (TSC) and tumor type-specific cytotoxicity (TTSC) as defined elsewhere [1]. The sensitivity to SLMC of Hu 456 significantly ($2P<0.01$, Wilcoxon's rank sum test for paired observations) exceeded that of T 24, when measured with lymphocytes from 24 control patients (group C, Fig. 2). When treated with lymphocytes derived from TCC patients the two TCC cell lines HU 456 and T 24 showed increased TSC and TTSC values as compared to cultures treated with control lymphocytes. However, the differences were not statistically significant.

The cytotoxicity of lymphocytes from patients suffering from interstitial cystitis (IC) to HU 609, representing normal urothelium, and SAOS 2 from osteosarcoma, has been compared with that of lymphocytes from 65 clinical control patients (Fig. 3). An increased cytotoxicity to HU 609 ($2P=0.018$, Mann–Whitney's rank sum test), but not to SAOS 2, was found in the IC patients.

ADCC

Positive ADCC tests were obtained with HU 456 cells treated with heat inactivated rabbit anti HU 456 serum in dilutions up to $1–2 \times 10^6$. Heat inactivated serum from one of 9 TCC patients was also active in the ADCC test as shown in Fig. 4 (I). Inhibition of SLMC and absence of detectable cytotoxicity was observed with four of five control sera and five of nine TCC patient sera (one is displayed in Fig. 4 II).

Inhibition of SLMC by Fab fragments

A relatively high lymphocyte concentration of 1.25×10^6 lymphocytes/ml was used in this experiment which included one TCC patient and one clinical control as lymphocyte donors (Fig. 5). A dose dependent inhibition of the cytoxicity was shown with Fab fragment concentrations from 7.8 to 125 μg/ml, but the inhibition vanished when the concentration was further augmented.

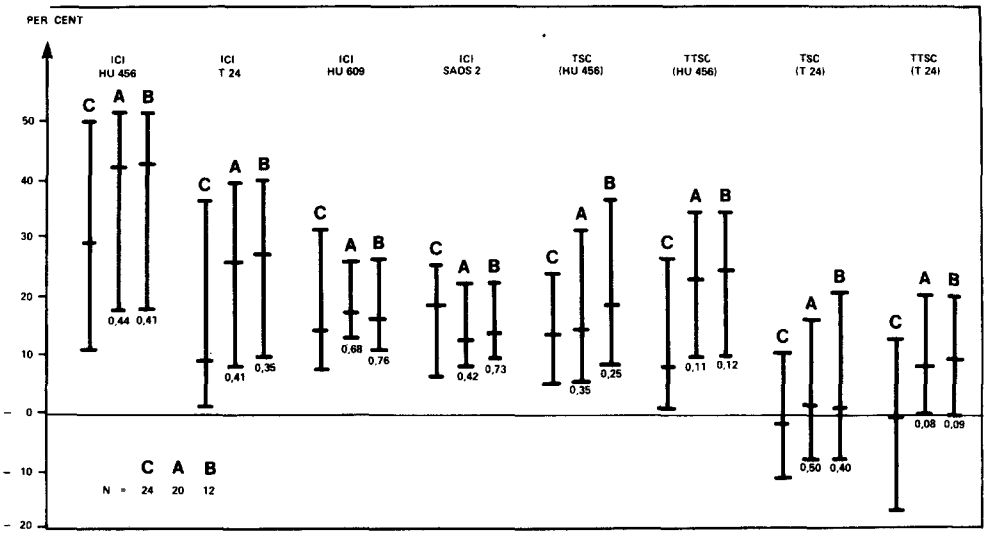

Fig. 2. Significantly increased sensitivity of TCC cell line HU 456 to SLMC in the microcytotoxicity assay as compared to TCC cell line T 24. Insignificantly increased TSC and TTSC of TCC-donor lymphocytes as compared to control lymphocytes against HU 456 or T 24 TCC target cells. ICI = integrated cytotoxicity index: The area delineated by the cytotoxicity titration curve, including lymphocyte concentrations $1.25 \times 10^6/\text{ml} - 0.04 \times 10^6/\text{ml}$.

$$TSC_{(HU\ 456)\ (or\ T\ 24)} = ICI_{(HU\ 456)\ (or\ T\ 24)} - ICI_{(HU\ 609)}$$

$$TTSC_{(HU\ 456)\ (or\ T\ 24)} = ICI_{(HU\ 456)\ (or\ T\ 24)} - ICI_{(SAOS\ 2)\ (osteosarcoma)}.$$

Lymphocyte donors: C, clinical control patients; A, all TCC patients investigated before treatment; B, TCC patients with non-invasive tumors of Gr. 2 to 3; N, number of patients in the group. Each patient was only tested once. The columns represent the distributions of ICI, TSC and TTSC values in each of the three groups when the uppermost and lowermost quartiles (25%) of values have been removed, and the small bar indicates the median. The level of significance of differences between the control group, C, and each of the groups A and B (2P) is indicated below the groups A and B (Mann–Whitney's rank sum test).

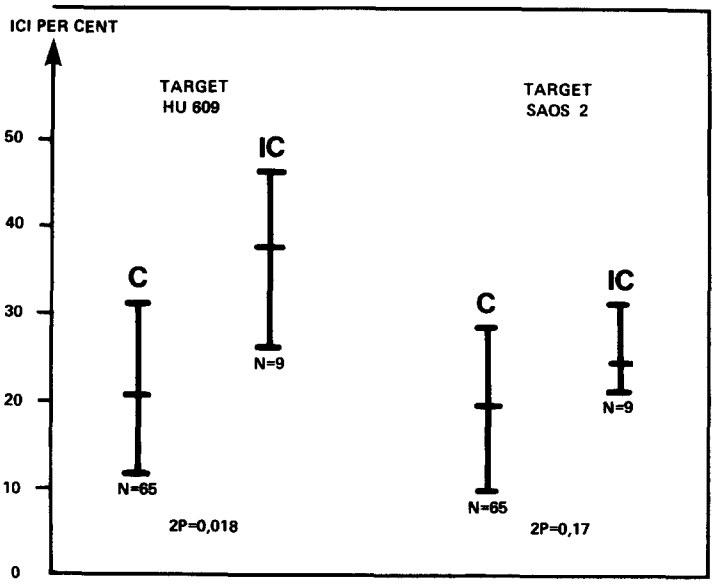

Fig. 3. Urothelium specific cytotoxicity of lymphocytes from patients with interstitial cystitis. Symbols as in Fig. 3, the columns represent distributions of ICI values of: C, 65 clinical control patients, each tested once; IC, 9 interstitial cystitis patients, each tested once; 2P, level of statistical significance of differences between cytotoxicities of groups C and IC (Mann–Whitney's rank sum test).

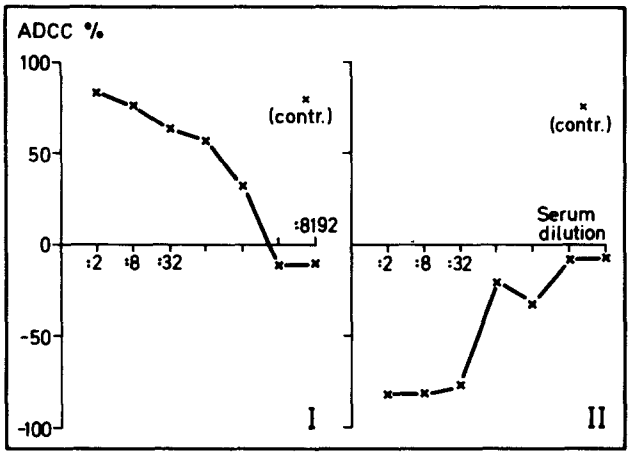

Fig. 4. Antibody activity against HU 456 in serum from a TCC patient in the ADCC test (I). *Inhibition of SLMC by serum from another donor* (II).

$$ADCC\% = \frac{\text{remaining cells with lymphocytes} - \text{remaining cells with lymphocytes + serum}}{\text{remaining cells with lymphocytes}} \times 100.$$

SLMC in these experiments averaged 50% × (*contr.*) = *ADCC*% *with rabbit anti HU* 456 *serum as positive control, dilution* 1:4000.

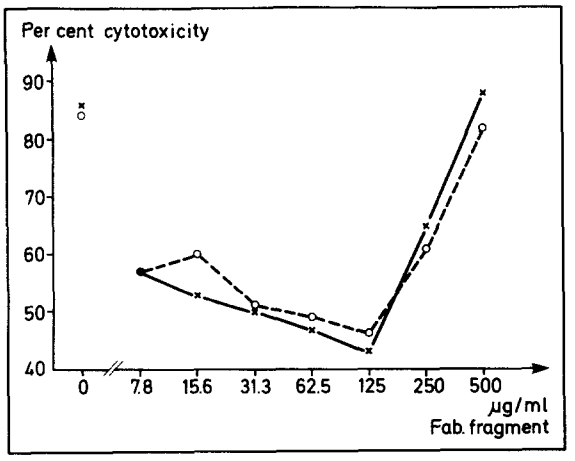

Fig. 5. Identical inhibition of cytotoxicity of lymphocytes from a TCC patient (———) *and a control patient* (------) *against HU* 456 *by Fab fragments of rabbit anti human Ig. Per cent cytotoxicity as in Fig.* 2.

DISCUSSION

HLA antigen expression in target cell cultures may be important for the lymphocyte-mediated cytotoxicity [18, 19]. Malignant transformation of cells *in vivo* may be associated by loss of isoantigens [20, 21], and the expression of antigens may change during cultivation [22]. Eventually HLA antigen expression of urothelium-derived cell lines from normal or malignant tissue might possibly differ from that of normal cells.

Demonstration of statistically significant cytotoxicity with the appropriate semispecific HLA antisera indicates that the present method is useful in HLA typing of cell lines. HLA antigens were present on slowly proliferating TCC cell lines but not on fast proliferating TCC lines or the squamous cell carcinoma line. HLA antigens were also demonstrated on the fast proliferating cell line HU 609 from normal urothelium before transformation but not after transformation. Some of the HLA antigens expected to be present on slowly proliferating TCC cells and one antigen on HU 609 could not be demonstrated by the present method. The possible loss of these antigens needs further investigation.

Short-term cultured melanoma cell lines were shown to be less susceptible to SLMC than cells grown *in vitro* for a longer time [23, 24]. This investigation shows that fast proliferating cell lines were more sensitive to SLMC than slowly proliferating cell lines. The significantly lower sensitivity to SLMC of T 24 compared with HU 456 may at least partly account for the discrepancy regarding specificity problems between cytotoxicity experiments performed in our laboratory [1, 2, 16] and the experiments of O'Toole *et al.* [7, 8]. Indications of tissue-specific antigen expression on HU 609 has previously been

observed when HU 609 was exposed to lymphocytes from patients who had undergone radiotherapy of the urinary bladder [2]. The increased cytotoxicity of lymphocytes from patients suffering from IC specifically directed against HU 609 indicated that bladder-specific antigens are present in this cell line, and further, that a cellular immune reaction may be of significance in the pathogenesis of IC.

The occasional demonstration of antibody activity against a TCC cell line in sera from TCC patients in a sensitive ADCC test is in accordance with others [6, 14]. In other cases inhibition of cytotoxicity by sera from TCC patients as well as controls could be demonstrated, indicating that the lack of positive ADCC reactions may at least in some instances be due to non-disease-specific blocking factors. Using purified Ig in such tests might eventually improve the possibility of detecting antibodies [15].

Dose-dependent inhibition of SLMC in the MA by Fab fragments is in agreement with data obtained by others in a ^{51}Cr-release assay [18], and may indicate that the mechanism behind the SLMC is an ADCC reaction.

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REFERENCES

1. M. Vilien, H. Wolf and F. Rasmussen, Titration of natural and disease-related cytoxicity of lymphocytes from bladder cancer patients. Correlation with tumor classification. *Cancer Immunol. Immunother.* **8,** 189 (1980).
2. M. Vilien, H. Wolf and F. Rasmussen, Follow-up investigations of bladder cancer patients by titration of natural and specific lymphocyte mediated cytotoxicity. Prognostic significance of specific reactivity. *Cancer Immunol. Immunother.*, in press.
3. M. Troye, M. Vilien, G. R. Pape and P. Perlmann, Cytotoxicity *in vitro* of blood lymphocytes from bladder cancer patients and controls to allogeneic or autologous tumor cells, derived from established cell lines or short term cultures. *Int. J. Cancer* **25,** 33 (1980).
4. M. Vilien, H. Wolf and F. Rasmussen, Cell lines from malignant and normal human urothelium I. Explantation and growth kinetics. In preparation.
5. M. Vilien, H. Wolf, F. Rasmussen, C. Hou-Jensen and C. Povlsen, Cell lines from malignant and normal human urothelium II. Morphology, chromosomes, heterotransplantation and transformation, In preparation.
6. J. Bubenik, P. Perlman, K. Helmstein and G. Moberger, Immune response to urinary bladder tumors in man. *Int. J. Cancer* **5,** 39 (1970).
7. C. O'Toole, P. Perlmann, B. Unsgaard, B. Moberger and F. Edsmyr, Cellular immunity to human urinary bladder carcinoma. I. Correlation to clinical stage and radiotherapy. *Int. J. Cancer* **10,** 77 (1972 *a*).
8. C. O'Toole, P. Perlmann, B. Unsgaard, L. E. Almgård, B. Johansson, G. Moberger and F. Edsmyr, Cellular immunity to human urinary bladder carcinoma. II. Effect of surgery and preoperative irradiation. *Int. J. Cancer* **10,** 92 (1972 *b*).
9. M. A. Bean, H. Pees, J. E. Fogh, H. Grabstald and H. F. Oettgen, Cytotoxicity of lymphocytes from patients with cancer of the urinary bladder. Detection by a [^{3}H]-proline microcytotoxicity test. *Int. J. Cancer* **14,** 186 (1974).
10. E. T. Bloom, R. C. Ossorio and S. A. Brosman, Cell-mediated cytotoxicity against human bladder cancer. *Int. J. Cancer* **14,** 326 (1974).
11. T. R. Hakala, P. H. Lange, A. Castro, A. Elliott and E. E. Fraley, Cell mediated cytotoxicity against human transitional cell carcinomas of the genitourinary tract. *Cancer (Philad.)* **34,** 1929 (1974).
12. M. Moore and N. Robinson, Cell-mediated cytotoxicity in carcinoma of the human urinary bladder. *Cancer Immunol. Immunother.* **2,** 233 (1977).
13. M. Troye, G. R. Pape, S. Paulie, M. Karlsson, P. Perlmann, H. Blomgren and B. Johansson, Disease-related *in vitro* cytotoxicity in patients with transitional cell carcinoma of the urinary bladder. Comparisons with other malignancies, acute cystitis and healthy donors. *Cancer Immunol. Immunother.* in press.

14. T. R. Hakala, P. H. Lange, A. E. Castro, A. Y. Elliott and E. E. Fraley, Antibody induction of lymphocyte-mediated cytotoxicity against human transitional-cell carcinomas of the urinary tract. *New Engl. J. Med.* **291,** 637 (1974).
15. M. Troye, Y. Hansson, S. Paulie, P. Perlmann, H. Blomgren and B. Johansson, Lymphocyte mediated lysis of tumor cells *in vitro* (ADCC), induced by serum antibodies from patients with urinary bladder carcinoma or controls. *Cancer Immunol. Immunother.* in press.
16. M. Vilien and H. Wolf, The specificity of the microcytotoxicity assay for cell-mediated immunity in human bladder cancer. *J. Urol.* **119,** 338 (1978).
17. M. Troye, P. Perlmann, G. R. Pape, H. L. Spiegelberg, I. Näslund and A. Gidlöf, The use of Fab fragments of anti-human immunoglobulin as analytic tools for establishing the involvement of immunoglobulin in the spontaneous cytotoxicity to cultured tumor cells by lymphocytes from patients with bladder carcinoma and from healthy donors. *J. Immunol.* **119,** 1061 (1977).
18. P. C. Doherty, D. Götze, G. Trinchieri and R. M. Zinkernagel, Models for recognition of virally modified cells by immune thymus-derived lymphocytes. *Immunogenetics* **3,** 517 (1976).
19. E. Goulmy, A. Termijtelen, B. A. Bradley and J. J. van Rood, Y-antigen killing by T cells of women is restricted by HLA. *Nature* (*Lond.*) **266,** 544 (1977).
20. E. Dabelsteen and J. J. Pinborg, Loss of epithelial blood group substance A in oral carcinomas. *Acta pathol. microbiol. scand.* Sect. A. **81,** 435 (1973).
21. I. Davidsohn, R. Stejskal and P. Lill, The loss of iso-antigens, A, B and H in carcinoma of the urinary bladder. *Lab. Invest.* **28,** 382 (1973).
22. C. Sorg, J. Brüggen, E. Seibert and E. Macher, Membrane-associated antigens of human malignant melanoma IV: Changes in expression of antigens on cultured melanoma cells. *Cancer Immunol. Immunother.* **3,** 259 (1978).
23. J. E. de Vries and P. Rümke, Tumor-associated lymphocyte cytotoxicity superimposed on 'spontaneous' cytotoxicity in melanoma patients. *Int. J. Cancer* **17,** 182 (1976).
24. E. Kristensen and R. Reimann, Direct cytotoxicity test applied to patients in stage I and II of malignant skin melanoma. *Cancer Immunol. Immunother.* **2,** 49 (1977).