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Antigen(s) on Human Transitional Cell Carcinoma Detected by a Xenogeneic Antiserum

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Summary. In studies concerning human bladder cancer, antisera were raised in rabbits against human tumours, normal tissue, and cell lines derived from human tumours and were analysed by absorption and complement dependent microcytotoxicity tests. No significant selective cytotoxicity was discernible with any unabsorbed antisera. After absorption of A53, (an antiserum against the transitional cell carcinoma derived cell line T24) with peripheral blood cells and normal adult tissues, it was cytotoxic to two bladder cancer cell lines (T24 and J82) but not to four other cell lines. This activity was removed by absorption with each of two specimens of transitional cell carcinoma but not by normal bladder and by absorption with T24 or J82 but not with four other non-bladder cell lines. This functional specificity for transitional cell carcinomas could be due to a tumour associated antigen, a significant quantitative difference between tumour and normal cells, or an embryonic specificity re-expressed on the tumour. Further experiments are necessary to investigate these alternatives.

Key words: Antigens - Transitional cell carcinoma - Xenogeneic antiserum

Abbreviations used: TAA = tumour associated antigen(s); NRS = normal rabbit serum; MEM = Eagle's minimal essential medium and additives; LuLiKi = mixture of homogenates of human lung, liver and kidney; TCC = transitional cell carcinoma; NBl = normal bladder.

Identification of antigens associated with human tumours is a major problem in tumour immunology. Detection of tumour specificity in assays for cellular immunity (13) are being challenged by reports of non-tumour related activity (23). Antibody against tumour associated antigens in serum from patients has been detected using various serological assays (12, 16, 19). This approach is also complicated by non-tumour associated reactivities (3, 4, 14). A third approach to the detection and identification of human tumour associated antigens (TAA) is preparation of xenogeneic antisera. Although such antisera will invariably contain a complexity of anti-human and anti-tissue antibodies in addition to any anti-TAA, the antisera can be obtained in quantity. The ability to obtain a large and easily stored amount of a potential reagent for serological analysis is a definite advantage. An excellent example of the usefulness of this approach is provided by the immuno- and biochemical characterization of carcinoembryonic antigen (9).

The present study was undertaken to determine if rabbit antisera could be used to recognise other human TAA. Complement dependent cytotoxicity assays and absorption tests were used to analyse antisera prepared against cell culture lines and preparations of tissue specimens. Transitional cell carcinoma of the urinary bladder was selected for initial emphasis in this study since observations by many investigators indicate that TAA maybe detected by other means (1, 5, 7).

MATERIALS AND METHODS

Antisera

Antisera were supplied by Drs. R. Akeson and H. E. Weimer, Department of Microbiology and Immunology, University of California,

Table 1. Major antisera used in present study

Serum	Antigen
A24	Particulate portion of homogenised lung tumour specimen, Lu-1
A28	HT29M cell culture line derived from colon carcinoma
A46	Particulate portion of homogenised bladder cancer specimen, Bl-1
A48	T24 cell culture line derived from transitional cell carcinoma of bladder
A49	MAC21 cell culture line derived from bronchogenic adenocarcinoma
A53	T24
A85	Particulate portion of homogenised normal tissues with three booster innoculations of homogenised normal bladder

Table 2. Target cells for cytotoxicity assay and/or cells used for absorption

Line designation[a]	Tissue derivation	
ChaGo	Pleomorphic undifferentiated carcinoma of the lung	22
FL	Amnion	10
HT29	Colon carcinoma	3
J82	Bladder (transitional cell) carcinoma	4
MAC21	Well-differentiated mucoid adenocarcinoma of the lung	8
T24	Bladder (transitional cell) carcinoma	6
4265	Lymphoblastoid cell line from blood of a patient with chronic myelogenous leukemia	18
LSTRA	Murine (BALB/c) moloney sarcoma	17

[a]All lines used in this study were shown to be mycoplasma-free.

Los Angeles. They were prepared by immunization of New Zealand white female rabbits (> 3 kg) by a modification of the protocol of Kim and Reif (15). Tissue culture cells ($5\text{-}10 \times 10^7$) were injected intravenously three times at biweekly intervals and blood was collected seven and ten days after the last injection. Particulate homogenates of tissue were emulsified with Freund's complete adjuvant and injected intradermally into the toe pads and abdominal skin. Table 1 lists the antigens and designations of the major sera used in this study. Many other antisera prepared against various tumour or normal tissue homogenates or extracts were also screened, but those studied in depth provided the information presented here. Pooled normal rabbit serum (NRS) was used as a control. All sera were stored at -70° C.

Cytotoxicity Test

The cytotoxicity test was performed as described previously for human serum (2). Relevant target cells are listed in Table 2. The medium used was Eagle's minimal essential medium with Earle's salts (Gibco, San Jose, Ca.), with 10% fetal calf serum (Reheis, Armour, Phoenix, Az.), 1% glutamine (Gibco), 1% fungizone (Gibco), and 50 µg/ml Gentamycin (Schering, Kenilworth, N.J.) (MEM). Briefly, 100-200 cells were allowed to adhere for 18-40 hours at 37° C in a 5% CO_2 humidified atmosphere to the bottom of Histoplates (Microbiological Associates, Los Angeles, Ca.) or Microtest I plates (Falcon 3034, Oxnard, Ca.). Media was aspirated and 10 µl of various dilutions of sera in MEM were added to test wells in triplicate. Test sera were heated at 56° C for 30 minutes prior to use. Plates were incubated one hour at 37° C in a 5% CO_2, humidified atmosphere. The test sera were aspirated and 10 µl of pooled normal rabbit serum diluted 1 : 4.5 or 1 : 5 in veronal buffered saline (pH 7.4) was added as a source of complement. After a 2 h incubation, each plate was flooded with 6 ml of MEM and incubated overnight to allow dead cells to detach from the plastic. Plates were washed twice, fixed in methanol, and stained with Giemsa's stain. The 50% cytotoxic endpoint titres were assessed compared to identical dilutions of NRS in adjacent wells of the same plates.

Absorptions

Antisera were absorbed at indicated dilutions (Table 3) in Earle's balanced salt solution with 10% fetal calf serum. All preparations used

Table 3. Preparations used for absorption of sera

Antigenic Preparation		Ratio
Designation	Composition	Serum:Antigen
PBC	Peripheral blood cells including both erythrocytes and leukocytes. PBC obtained from at least 3 donors (1 each of blood group A, B, and 0) were combined.	1:1 (v/v)
LuLiKi	Normal lung, liver and kidney obtained 8-12 hours post-mortem. Tissues were homogenised and combined in equivalent amounts of protein for (a); or 1:2:10 (v/v/v) respectively according to availability for (b).	(a) 400 µl:2 mg Lowry protein or 4:1 (v/v) (b) 2:1 (v/v)
TCC_1[a] TCC_2[a]	Transitional cell carcinomas 1 and 2. Specimens were obtained post-operatively and all suspensions were prepared with 0.25% trypsin for 30 minutes at room temperature.	200:15 (v/v)
NBl_1[a]	Normal bladder epithelium obtained at cystectomy following 4000 R irradiation. Absence of tumour confirmed by pathology.	200:15 (v/v)
T24, FL	Cell culture lines grown in quantity and obtained by trypsinisation.	10:1 (v/v)
4265	Suspension cell culture line	10:1 (v/v)
LSTRA	Murine ascites tumor	10:1 (v/v)

[a]Confirmed by pathology. TCC_1 was an ureteric and TCC_2 a bladder tumour.

for absorption were washed at least three times with phosphate buffered saline prior to use; if trypsin was used in preparation, they were allowed to "recover" at least 1 h before absorption. Depending on quantities of sera absorbed, absorptions were carried out in 15 ml plastic conical centrifuge tubes or in 0.4 ml Microfuge tubes (Beckman Instruments, Fullerton, Ca.). Sera were mixed with the absorbing antigen, incubated for 1 h at 4° C and recovered by centrifugation. Each serum aliquot was absorbed with one or more cell preparations as described in Table 3 and tested for cytotoxicity together with the same dilution of the unabsorbed antiserum.

For specimens obtained post-operatively and used for subsequent absorptions, histological diagnosis was reconfirmed by pathology. Of the two transitional cell carcinomas, one was a primary ureteric tumour and one was a bladder cancer.

RESULTS

Activity of Unabsorbed Antisera

Selection of xenogeneic antisera for absorption studies was made after preliminary screening. A large number of antisera against several human carcinomas were tested against a variety of cell lines. Antisera showing potentially selective killing or high titres and also "control" antisera were chosen for absorption. Reproducibility of serum titres was ascertained in repeated tests. Unabsorbed sera did not generally demonstrate significant differential reactivities. Titres were normally higher with antisera raised against cell lines (~ 1:1000-2000) than with antisera raised against comparable non-cultured tissues (~ 1:100) regardless of tumour system.

Table 4. Absorption analyses with cell culture lines

Serum	Dilution	Immunogen	Absorption	Target cells[a] T24	J82	FL	MAC21	HT29M
A28	1:100	HT29M	0	1:1600	NT[b]	1:400	1:800	>1:3200
			PBC	1:400-800	NT	1:200-400	1:400	≥1:3200
			PBC + LuLiKi[c]	-[d]	NT	-	-	1:200-400
A49	1:100	MAC21	0	1:800	NT	1:800	1:800-1600	>1:3200
			PBC	1:200-400	NT	1:400	1:800-1600	>1:3200
			PBC + LuLiKi	≤1:100	NT	-	1:800	>1:3200
A53	1:100	T24	0	1:1600	1:800	1:800	1:800	NT
			PBC	1:400	1:400	1:100	1:200	NT
			PBC + LuLiKi	1:200	1:100-200	-	-	NT
A85	1:10	Normal bladder	0	>1:640	NT	1:640	NT	NT
			PBC	-	NT	-	NT	NT

[a] See Table 2 for description of target cells.

[b] Not tested.

[c] This absorption with normal lung, liver, and kidney was carried out according to protocol a in Table 3.

[d] Indicates no detectable cytotoxicity at lowest dilution used.

Preliminary Absorptions

In order to reduce non-specific activity against normal human tissue components, sera were first absorbed with normal human tissues. Table 4 gives results of tests of some of these absorbed sera. Although in some sera, e.g., A28 and A85, the detectable non-specific activity could be completely absorbed, clearly further absorptions were needed with the other antisera. All tests and absorptions were performed in 10% fetal calf serum to remove anti-fetal calf serum antibodies in the antisera prepared against cell culture lines.

Specific Absorption Analysis

In order to conclude successful absorption techniques of antisera against cell lines, it is necessary to demonstrate that the activity against the immunizing target cell lines can be completely removed by absorption with the immunizing antigen. Table 5 gives results of absorption analysis of two anti-T24 sera. The results with A53 clearly show that only T24 removes activity completely. Furthermore, although all human cell lines removed non-specific activity against FL, only T24 removes cytotoxic antibody against T24 and J82. This indicates a cross reactivity between the two bladder cancer cell lines. The LSTRA control shows that non-specific absorption did not occur with A53 under the experimental conditions employed. With the serum A48, however, no specific reactivity could be detected: all activity was absorbed with all cell lines with some absorption even by the LSTRA control.

Antisera were diluted 1 : 10 for further absorption analysis. Results of absorption of A53 shown in Table 6 using both PBC and LuLiKi for preliminary absorptions confirm and extend findings in Table 5. In this experiment absorbed sera were tested down to 1 : 10 dilution. At this level some activity was still detectable against non-bladder cancer cell lines but this was removed by absorption with non-bladder cancer cell lines. As before, only T24 removed activity for T24 and J82.

Results of absorbing A53, A46, A49, and A24 first with preliminary material and then with fresh transitional cell carcinoma or normal bladder tissue is shown in Table 7. Most experiments were repeated at least twice.

Table 5. Absorption analyses with cultured cells of two antisera against T24

Serum	Dilution	Absorbed with	Target cells[a]		
			T24	J82	FL
A53	1:100	0	1:1600	1:3200	1:1600
		PBC	1:400	1:800	1:400
		PBC + T24	-[b]	-	-
		PBC + FL	1:200	1:100	-
		PBC + 4265	1:800	1:800	-
		PBC + LSTRA[c]	1:800	1:800	1:400
A48	1:100	0	1:1600	1:400	1:400
		PBC	1:1600	1:400-800	1:200
		PBC + T24	-	-	-
		PBC + FL	-	-	-
		PBC + 4265	-	-	-
		PBC + LSTRA[c]	1:200	-	-

[a]See Table 2 for description of target cells.

[b]No detectable activity at 1:100 serum dilution, the lowest dilution used.

[c]LSTRA is a murine tumour used as a control.

Table 6. Absorption of A53 with PBC, LuLiKi and cultured cell lines[a]

Absorb with	Test against					
	T24	J82	FL	ChaGo	MAC21	HT29M
0	>1:320	>1:320	≥1:320	>1:320	>1:320	>1:320
PBC	≥1:320	>1:320	≥1:320	1:320	>1:320	1:320
PBC + LuLiKi + T24	1:40	1:80	1:10	1:10	-[b]	<1:10
" + " + FL	1:20	NT	-	-	NT	-
" + " + ChaGo	1:20	NT	-	-	NT	-
" + " + MAC21	1:40-80	1:40	-	NT	-	NT
" + " + HT29M	1:20	1:40	-	-	-	-

[a]Tests begun at dilution of 1:10.

[b]Indicates no detectable activity at lowest dilution of 1:10.

NT = Not tested.

Table 7. Absorption analyses with fresh tissue specimens

Serum/ Immunogen	Dilution						Bladder cancers		Amnion	Lung cancers	
		PBC	LuLiKi[a]	TCC_1	TCC_2	NBl_1	T24	J82	FL	MAC21	ChaGo
							> 1:640	> 1:640	> 1:640	1:640	≥ 1:640
		+					1:320-640	1:320-640	1:80-160	1:80	1:320
		+	+				1:40-80	1:40	-[b]	-	-
A53/T24	1:10	+	+	+			-	-	-	-	-
		+	+		+		-	-	-	-	-
		+	+			+	1:40	1:20	-	-	-
							1:20	1:20-40	1:20	≤ 1:20	1:40
		+					-	1:20	-	-	-
A46/Bl-1	1:10	+		+			-	-	-	-	-
		+			+		-	-	-	-	-
		+				+	-	-	-	-	-
							> 1:640	> 1:640	> 1:640	1:640	≥ 1:640
		+					1:320	> 1:640	1:320	1:160	1:320
		+	+				1:80	1:160	1:160	1:80	1:160-320
A49/MAC21	1:10	+	+	+			-	1:160	1:320	1:80	1:160
		+	+		+		-	-	-	1:40-80	1:160
		+	+			+	-	1:40-80	1:80	1:40	1:160-320
							1:160	1:320	1:80	1:40-80	1:160
		+					1:20-40	1:80	≤ 1:20	1:20	1:80-160
A24/Lu-1	1:10	+		+			1:20	1:40	< 1:20	-	1:40
		+			+		-	-	-	-	1:20-40
		+				+	1:20	1:40-80	-	-	1:40

[a] Absorption according to protocol b in Table 3.

[b] Indicates no detectable cytotoxicity at 1:20.

Confirming results shown in Table 6, absorption with PBC and LuLiKi decreased activity to $< 1:20$ in A53 (anti-T24) against FL, MAC21, and ChaGo while leaving detectable activity only against the two bladder cancer target cell lines T24 and J82. The remaining activity was removed by absorption with each of two transitional cell carcinomas but not by normal bladder tissue.

Table 7 shows that absorption of A49 with PBC, LuLiKi and TCC left cytotoxic activity against only ChaGo and MAC21 (lung adenocarcinoma derived), though this information is quite insufficient to extrapolate to tumour associated activity in the serum. Absorption of A24 (anti-Lu-1) or A46 (anti-Bl-1) disclosed no potentially tumour associated activities in these sera. This was generally found to be true with 17 other antisera raised against human tumours including carcinomas of the bladder, breast, colon and lung.

DISCUSSION

Serological analyses of xenogeneic antisera present an interesting and potentially practical approach to human tumour immunology. Rabbit antisera against human tumours, normal tissues, or cell culture lines were investigated using a complement dependent cytotoxicity assay and absorption analysis. Of particular interest was an antiserum, A53, prepared against T24, a cell line derived from a transitional cell carcinoma of the bladder. Absorption of A53 with PBC and LuLiKi (Table 7) or PBC, LuLiKi, and other cell culture lines (Table 6) left activity against only the two target cell lines derived from bladder cancers: T24 and J82. This activity could be completely abolished by absorption with T24 or each of two different specimens of transitional cell carcinomas, but not with non-malignant bladder or non-bladder cancer cell lines. The selective cross absorption of T24, J82, TCC_1 and TCC_2 suggests the presence of a tumour associated antigen. Alternative explanations include quantitative antigenic differences or presence of fetal antigens; more extensive studies will be necessary for definitive decision. Nevertheless, A53 was made at least functionally specific for transitional cell carcinomas.

Furthermore, tumours of the bladder (TCC_2) and ureter (TCC_1) were equally efficient for absorption of A53. Although more experiments are needed for definitive conclusion, the detected specificities were shared by transitional cell carcinomas used in this study regardless of anatomical location.

Previous studies (1, 5, 7, 11, 12, 20, 21), indicated that T24 possesses antigen(s) associated with bladder cancer which can be detected using assays for cellular immunity. Present data support this; however, there is no current evidence that the serological and cell-mediated tests are detecting the same antigen(s).

The immunological cross reactivity of the transitional cell carcinomas used in this study may reflect a quantitative rather than qualitative difference in the cells. This question can be answered by quantitative absorption analysis. The possibility of the reappearance of fetal antigens can also be approached by appropriate absorptions. It is unlikely that the differences between the bladder cancer and other cell lines are referable to different sensitivities to antiserum and complement: T24, J82, FL, MAC21, HT29M, and ChaGo are all similarly vulnerable to unabsorbed antisera. Furthermore, there appears to be little change in sensitivity of these cultures with passage.

The implications of this study are far reaching, despite certain inherent difficulties. Major yet surmountable problems exist due to the lack of consistent production of antisera with the same spectra of reactivities (e. g., A53 versus A48 in this study). Appropriate and adequate absorption of antisera also presents logistical problems such as the procurement of correct absorbing material and choice of dilution of antisera. Nevertheless, if xenogeneic immunisations with cell culture lines can be used for preparation of at least functionally defined antibody with anti-tumour activity, further characterisation of these antigens (TAA? Quantitative? Fetal?) will be possible. There also exists the possibility to prepare large quantities of reagents as an homogeneous reagent for clinical/diagnostic applications.

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