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H-2 Typing of RBL-5, TLX/9 Tumour Cells and L929 Fibroblast *

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The H-2 specificities in two different H-2^b tumour cells (TLX/9 and RBL-5) and a fibroblast line L929 (H-2^k) have been studied.

Tumour cells were passaged intraperitoneally in syngeneic mice and fibroblast was kept *in vitro* at 36° C. The typing was performed by means of a postlabelling microradioassay for actively growing cells and 28 H-2 alloantisera of restricted specificity defining private H-2K, H-2D and public antigens.

Unexpected reactions in disagreement with H-2 genetics were detected in both tumours but not in fibroblast line. The latter shed C-type particles whereas RBL-5 did not. The cytotoxicity of the alloantisera was quantitatively absorbed by normal lymphoid cells, althrough some extrareactivities were due to other antibodies present in the sera. RBL-5 grew in mice of different H-2 haplotypes with the same intensity as in syngeneic animals.

There are increasing evidences pointing to the possibility of tumour specific transplantation antigens (TSTA) being foreign histocompatibility antigens (2, 3, 6). Despite the mechanism of this new antigenicity is still controversial (1, 4) the typing of tumour cell lines of different ethiology with a wide panel of anti H-2 sera is an appropriate way to detect and characterize anomalous reactions not in accordance with genetic expectations.

In this report it is shown the pattern of reactivity of 28 H-2 alloantisera with two H-2^b tumour cells and a fibroblast line (H-2^k) using a postlabelling radioassay. Finally we have tried to analize some of the citotoxicity reactions by absorptions with normal lymphoid cells and studied the pattern of growth of RBL-5 in mice of different H-2 haplotypes.

Materials and Methods

Tumour cells. The following H-2^b tumours were used. RBL-5 (a Rauscher vi-

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rus induced leukemia from C57BL/6 mice, provid by Prof. Schirrmacher, Cancer Research Center, Heidelberg); TLX/9 (X rays induced leukemia from C57BL/6 provided by Prof. Alexander, Chestter Beatty Research Institute, Sutton, England). They were kept by intraperitoneal injections in our laboratory in syngeneic C57BL/6 mice.

Fibroblast line. A fibroblast cell line (L929, H-2^k from C3H mice provided by Prof. Heat, London), was maintained *in vitro* by culturing in Falcon flask with Eagle Medium (MEM) 2,5 % of sodium bicarbonate, 100 mg/ml of cloxac/ampicillin, supplemented with 10 % of fetal calf serum (Difco) and 1 % glutamine (Gibco). When using for typing, they were detached from the flask by shaken with Pasteur pipettes and without trypsin. They were processed later in the same way as the tumour cells.

Mice. The following congenic mice of our colony were used: C57BL/6 (H-2^b), B10 (H-2^b), B10. BR (H-2^k) and B10. HTT (H-2^{t3}). They were obtained from Prof. Festenstein, Tissue Immunology Unit, London and were used for passage the tumour cells (C57BL/6) and to perform absorptions of H-2 alloantisera with positive or negative lymphoid cells for a particular specificity.

Antisera. A panel of 28 H-2 alloantisera (kindly provided by Dr. Ray, NIH, Bethesda, Maryland) of restricted specificity defining private H-2K, H-2D or public H-2 antigens were used to test the presence or absence of H-2 specificities on RBL5, TLX/9 and L929 fibroblast. Poliespecific anti H-2 sera were raised by weekly intraperitoneal injections of 10^7 spleen cells for seven weeks. In this way it was obtained anti H-2^k (C57BL/6 anti B10.BR), anti H-2^b (CBA/H anti C57BL/6) and anti H-2^d (CBA/H anti BALB/c). Normal mouse serum was obtained from C57BL/6.

Typing technique. A microradioassay was used for typing actively growing cells. It combines antibody complement treatment of target cells following by measuring ^{14}C-thymidine uptake in a postlabelling assay. Details of the procedure have been published elsewhere (4).

Absorption procedures. To characterize expected and unexpected reactions, H-2 alloantisera were absorbed with positive and negative lymphoid cells for a particular specificity. Spleen and lymph node cells were used as absorbing cells. Quantitative absorptions were performed increasing the number of absorbing cells from 10^7 to 5×10^7, keeping constant the amount of antiserum (50 μl of 1/10 dilution).

Monitoring of in vivo growth of RBL-5 in mice of different H-2 haplotypes. 10^7 RBL-5 tumour cells were injected subcu-

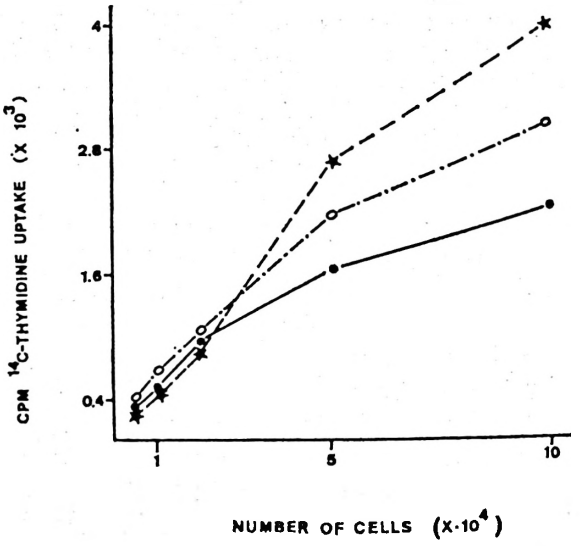

Fig. 1. *^{14}C-Thymidine uptake by RBL-5 (----), TLX-9 (—·—·—) and L929 fibroblast (—) with increasing number of cells.*
Dose response curve.

taneously into groups of mice of the following strains: C57BL/6 (H-2^b), B10 (H-2^h), BALB/c (H-2^d), CBA (H-2^k), B10.HTT (H-2^{t3}). Tumour growth was assesed by palpation every four days.

Results

^{14}C-thymidine uptake by RBL5, TLX/9 tumour cells and L929 fibroblast. In order to select the number of cells required

Citotoxic activity on anti H-2 sera against RBL5, TLX/9 lymphomas (H-2^b) and L929 fibroblast (H-2^k)

2×10^4 tumour cells or L929 fibroblast in 50 μl of culture medium, were incubated in round bottom wells of sterile microtitre plates with 1 μl of antiserum for 1 h. and then 30 min. With 50 μl of prediluted 1/10 rabbit complement at room temperature. 10 μl of ^{14}C-thymidine (4 μCi ml^{-1}) were added to the plates for a further 16 h. The results are expressed as the percentage reduction in thymidine uptake in test samples compared with control samples with complement and no antibody.

Antisera against	RBL5 (H-2^b)		TLX/9 (H-2^b)		L929 (H-2^k)	
	^{14}C-Thymidine uptake	% Reduction	^{14}C-Thymidine uptake	% Reduction	^{14}C-Thymidine uptake	% Reduction
—	1,248 ± 84		1,185 ± 126		1,280 ± 97	
H-2.1	711 ± 113	43	900 ± 118	24	38 ± 16	97 *
H-2D.2	74 ± 28	94 *	71 ± 33	94 *	1,254 ± 235	2
H-2.3	936 ± 89	25	1,230 ± 218	0	166 ± 43	87 *
H-2D.4	1,415 ± 236	0	1,199 ± 183	0	1,315 ± 129	0
H-2.5	87 ± 118	93 *	225 ± 96	81 *	25 ± 10	98 *
H-2.8	698 ± 149	44	1,345 ± 234	0	192 ± 86	85 *
H-2.9	312 ± 89	75 *	367 ± 112	69 *	1,295 ± 241	0
H-2.11	898 ± 204	28	912 ± 147	23	77 ± 23	94 *
H-2D.12	985 ± 187	21	924 ± 181	22	1,309 ± 187	0
H-2.13	1,173 ± 215	6	1,102 ± 238	7	1,062 ± 193	17
H-2K.15	923 ± 138	26	1,149 ± 164	3	1,336 ± 219	0
H-2.16	224 ± 75	82 *	509 ± 23	57 *	960 ± 133	25 *
H-2K.17	1,060 ± 173	15	639 ± 143	46	1,139 ± 192	11
H-2.18	1,048 ± 132	16	663 ± 138	44	1,229 ± 214	4
H-2K.19	736 ± 148	41	687 ± 189	42	N.D.	
H-2K.19b	162 ± 98	87 *	225 ± 47	81 *	166 ± 47	87
H-2K.20	174 ± 49	86 *	165 ± 28	86 *	755 ± 183	41
H-2.21	349 ± 137	72 *	533 ± 73	55 *	N.D.	
H-2K.23	911 ± 84	27	1,359 ± 185	0	243 ± 84	81 *
H-2.25	686 ± 119	45	438 ± 66	63	13 ± 7	99 *
H-2.28b	162 ± 37	87 *	82 ± 21	93 *	1,024 ± 192	20
H-2.29	149 ± 63	88 *	154 ± 53	87 *	N.D.	
H-2D.30	511 ± 123	59	355 ± 108	70	1,216 ± 237	5
H-2K.31	1,085 ± 231	13	1,204 ± 239	0	1,418 ± 119	0
H-2D.32	736 ± 148	41	912 ± 148	23	102 ± 52	92 *
H-2K.33	87 ± 26	93 *	142 ± 71	88 *	1,088 ± 176	15
H-2.35	74 ± 31	94 *	94 ± 37	92 *	1,293 ± 249	0
H-2.36	386 ± 84	69 *	296 ± 84	75 *	N.D.	
ATH anti ATL	661 ± 158	47	793 ± 123	33	1,216 ± 135	5
Thy 1.2	174 ± 65	86	130 ± 48	89	1,329 ± 223	0
Ly 4.2	574 ± 168	54	628 ± 131	47	1,378 ± 132	0
CBA/H anti C57 B1/6	87 ± 21	93 *	71 ± 20	97 *	1,230 ± 199	4
C57B1/6 anti B10.Br	1,198 ± 115	4	663 ± 154	44	75 ± 12	94

* Expected positive reactions for H-2^b and H-2^k haplotypes according to the NIH catalogue.

for the microradioassay, the ^{14}C-thymidine uptake by increasing numbers of RBL5, TLX/9 and L929 cells, was determined. 20.000 cells gave around 10^3 cpm and this concentration was selected for the standard assay.

H-2 typing of RBL5 (H-2^b), TLX/9 (H-2^b) and L929 fibroblast (H-2^k). Table I shows the results obtained with 28 H-2 alloantisera of restricted specificity (main specificity is indicated) polispecific anti H-2^d and anti H-2^k sera, anti θ serum (AKR anti C3H), anti Ly 4.2 (BALB/c × DBA/2 anti B10.D2) and anti Ia serum (A.TH anti A.TL). The results are expressed in cpm of ^{14}C-thymidine uptake by the different type of cells and by the percentage of reduction when the uptake is compared with the results obtained with the complement alone (rabbit serum).

RBL5 and TLX/9 seems to be θ posi-

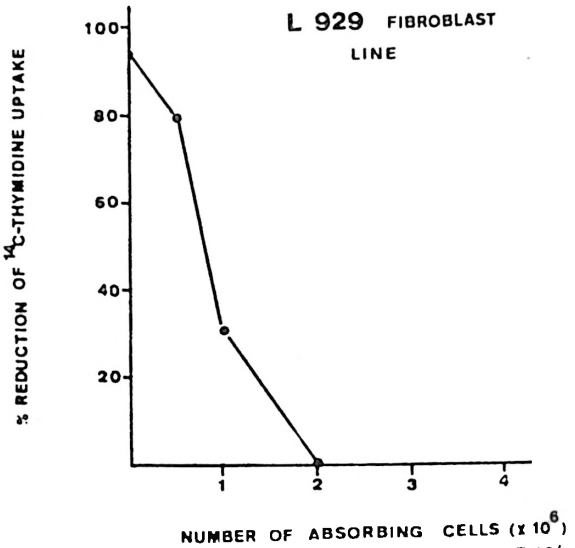

Fig. 2. *Quantitative absorption of serum D19b by B10.BR normal lymphoid cells.*
The citotoxicity of sera D19b on L929 fibroblast is absorbed with 2×10^6 B10.BR cells and must be due to an unidentified antibody present in this serum and not to anti H-2K19.

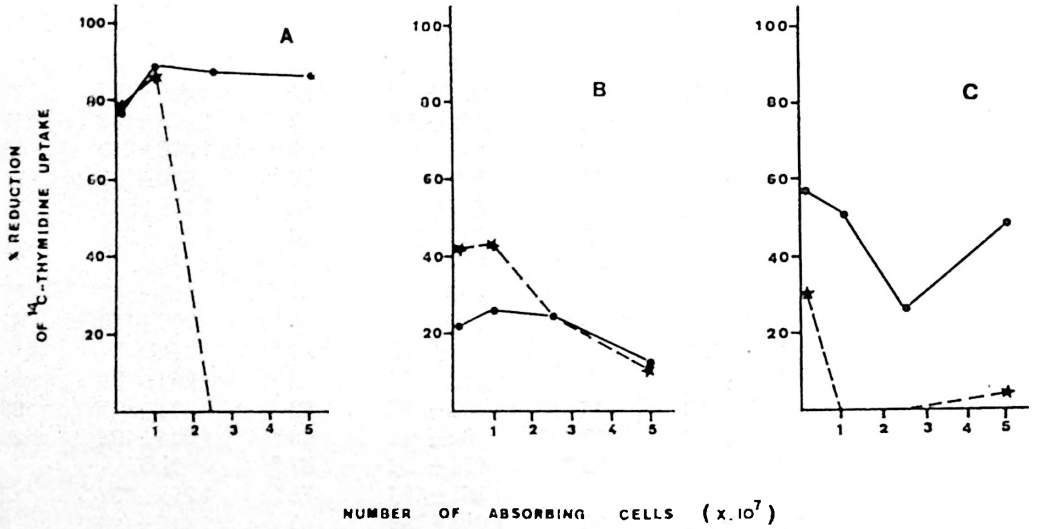

Fig. 3. *Citotoxicity of anti H-2.D2 (A), anti H-2.1 (B) and anti H-2D.30 (C) in RBL5 tumour cells (H-2^b) after quantitative absorptions with normal lymphoid cells from B10 (-----) and B10.HTT (——) mice.*
(A) The citotoxicity of anti H-2D.2 (a private specificity of H-2^b haplotypes) was absorbed as expected with 3.10^7 B10 cells (H-2D2 positive) and not by B10.HTT (H-2D2 negative). (B) The unexpected citotoxicity of anti H-2.1 on RBL5 (43 % reduction in ^{14}C-thymidine uptake) was absorbed better by B.10HTT (H-2.1 positive) than by B10 (H-2.1 negative). (C) The unexpected citotoxicity of anti H-2D.30 on RBL5 was absorbed completely by B10 (H-2D.30 negative) and not by B10.HTT (H-2D.30 negative) and must be due to an unidentified antibody different of anti H-2D.30 present in the serum and reacting with H-2^b haplotypes.

tive and Ia, Ly 4.2 negative tumours. Private specificities of H-2^b haplotypes (H-2D.2 and H-2K33) are normally expressed on RBL5 and TLX/9 (94 % and 93 % reduction in counts). Also public specificities H-2.5, H-2.28, H-2.29, H-2.35 and H-2.36 are also present as expected on both type of cells.

Abnormal citotoxicity reactions were also obtained with various anti H-2 sera because according to the NIH catalogue they contain other antibodies than the main specificity and killed H-2^b cells; this accounts for anti H-2.9, anti H-2.16, anti H-2.19^b, anti H-2.20 and anti H-2.21 (table I). Other intermediate or strong reactions cannot be interpreted so easily because according to the NIH catalogue these sera do not react with H-2^b cells.

In contrast L929 fibroblast behave quite normal when typing with the panel of anti H-2 sera. The citotoxicity was in accordance with normal H-2^k lymphoid cells.

Absorption of H-2 alloantisera with normal lymphoid cells of different H-2 haplotypes, to characterize expected and unexpected reactions. Serum D19^b Killed L929 fibroblast strongly (87 % reduction in counts). However H-2K,19 is a private specificity of H-2^{13} haplotypes. In other to know is the main specificity assigned to the serum was responsible for the killing, D19^b serum was absorbed with increasing numbers of B.10.BR lymphoid cells (fig. 2). It can be seen that the citotoxicity is totally removed with 2×10^6 cells.

The citotoxicity of anti H-2D.2 on RBL5 was absorbed as expected by 2.5×10^7 B10

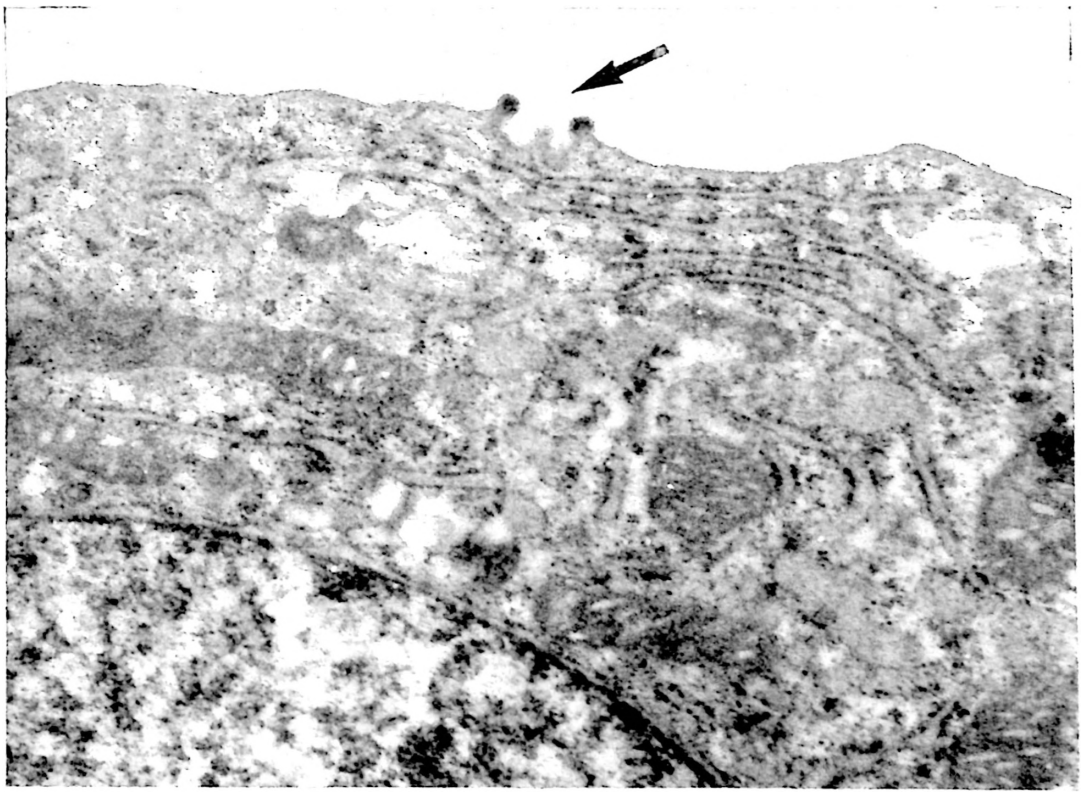

Fig. 4. *High power micrograph of L929 fibroblasts (×100,000).* Expression of C-type particles on the surface of L929 cells.

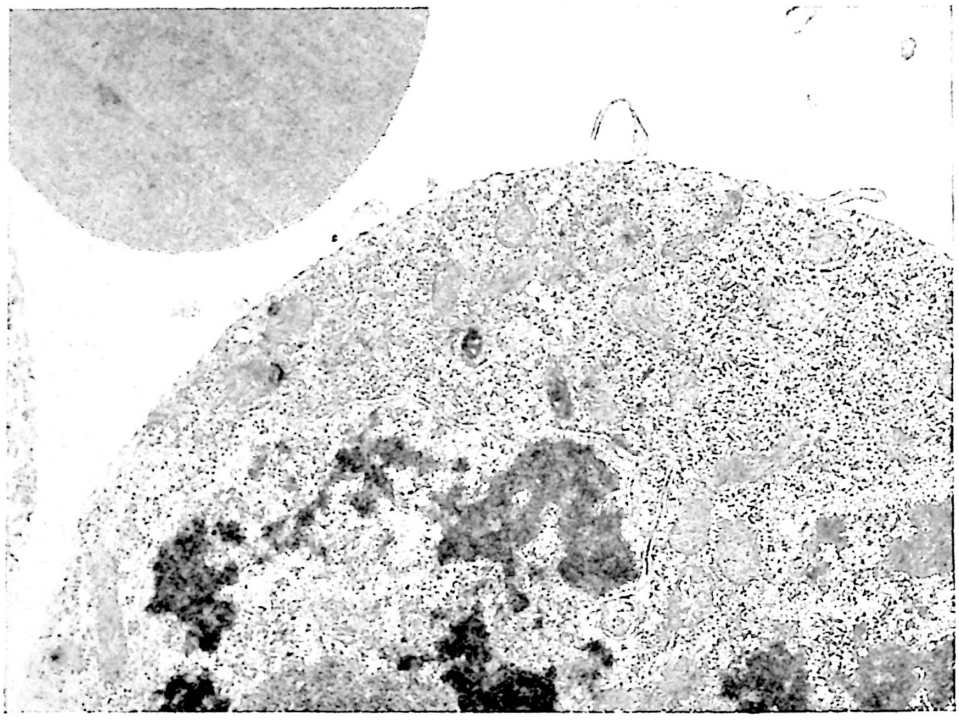

Fig. 5. *High power micrograph of RBL-5 tumour cell (× 60,000).* Absence of C-type particles in RBL5 leukemia.

($H\text{-}2^b$) lymphoid cells and not by B10.HTT (H-2D.2 negative) (fig. 3 *A*).

Fig. 3 *B* and *C* shows two examples of unexpected citotoxicity on RBL5 tumour cells: 43 % with anti H-2.1 and 59 % with anti H-2.30. An H-2.1 like specificity seems to be expressed on RBL5. Serum D1b was absorbed better by B10.HTT (H-2.1 positive) than by B10 (H-2.1 negative). However H-2D.30 is not present on RBL5 despite the citotoxicity obtained. Serum D30 was absorbed completely with 10^7B10 cells (fig. 3 *C*) and the citotoxicity on RBL5 must be due to an unidentified antibody present in the serum and reacting with $H\text{-}2^b$ haplotypes. Further more anti H-2D.30 also killed TLX/9 tumour cells (70 % reduction in counts).

Finally, when testing *in vivo* growth of RBL5 ($H\text{-}2^b$) in mice of different H-2 haplotypes it was found to growth with the same intensity in B10.HTT ($H\text{-}2^{b2}$), CBA/H ($H\text{-}2^k$), BALB/c ($H\text{-}2^d$) than in B10 and C57BL/6 (with the same H-2 haplotypes as the tumour cells), suggesting, that some of the potencial foreign H-2 like specificities expressed, in RBL5, could behave as tumour specific transplantation antigens (TSTA).

Experiments are in progress for typing various methylcolantrene induced sarcomas in congenic mice to see if there is a pattern of expression of foreign H-2 like specificities, or if it is a random process.

Discussion

Unexpected reactions of the NIH alloantisera with tumour cells have been reported by several authors (2, 6, 7). Nevertheless, the interpretation of the results are discordant among different groups.

The presence of foreign H-2 like specificities on the surface on tumour cells as

well as on virus infected cells have been pointed out recently (2, 3). A derepression hypothesis is required for it and implies a multigene H-2 system rather than the two locus model with alternative genes (alleles).

Another different interpretation for these unexpected results is the presence of anti MuLv antibodies in the NIH alloantisera that should kill tumour target cells expressing MuLv antigens and shedding C-type particles (5, 7).

From these results (table I) is evident that L929 fibroblast ($H\text{-}2^k$) behave as expected (no extrareactions) despite they are shedding C-type particles (fig. 4). In contrast RBL5 and TLX/9 ($H\text{-}2^b$) do have unexpected reactions (10 extrareactivities) and the former is not expressing C-type viruses (fig. 5). Absorption data show that, *a*) H-2D.2 is normally present in RBL5; *b*) An H-2.1 like specificity is expressed on RBL5; *c*) The citotoxicity of serum D30 on RBL5 is not due to anti H-2D.30 antibodies because it is completely absorbed by B10 normal lymphoid cells.

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Resumen

Se estudia la presencia de especificidades H-2 en dos tipos distintos de tumores $H\text{-}2^b$ (RBL-5 y TLX/9, respectivamente), así como en los fibroblastos L929.

Las células tumorales fueron pasadas intraperitonealmente en ratones singénicos y los fibroblastos mantenidos *in vitro* a 36° C. El tipaje se efectuó mediante un microrradioensayo diseñado para células que se multiplican activamente y 28 aloantisueros H-2 de especificidad restringida que definen antígenos privados H-2K, H-2D y antígenos públicos.

Reacciones inesperadas en desacuerdo con la genética del sistema H-2 se detectaron en ambos tumores, pero no en los fibroblastos. Estos eliminaron partículas virales tipo C mientras que el tumor RBL-5 no. La citotoxicidad de los aloantisueros fue cuantitativamente absorbida por células linfoides normales, aunque algunas extrarreactividades fueron debidas a otros anticuerpos presentes en el suero. El tumor RBL-5 creció en ratones de diferente haplotipo H-2 con la misma intensidad que en ratones singénicos, sugiriendo una relación entre extrarreactividades H-2 y TSTA.

Se discute la teoría de la derrepresión de genes H-2 para explicar estos resultados.

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