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Expression of H-2^b Alloantigens in a Variant of a Moloney Virus-induced YAC (H-2^a) Lymphoma

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Abstract—*Splenocytes of A(H-2^a), BALB/c(H-2^d) and C57BL/6(H-2^b) mice that were sensitized against several allogeneic normal lymphocytes and lymphoma cells in mixed lymphocyte cultures (MLC) and mixed lymphocyte–tumor cell cultures (MLTC) exhibited the expected cell-mediated cytotoxic responses against normal and tumor cells carrying the relevant alloantigens. In contrast, strong cytotoxic reactivity cross-reactive with C57BL/6 target cells was observed when a variant of YAC lymphoma (YAC-b) of strain A mice were employed either as sensitizers or as targets. Furthermore, this 'unpredicted' cross-reactivity was also obtained with syngeneic responder splenocytes sensitized to YAC-b cells. The possibility that the nonspecific cytotoxicity was caused by activation of natural killer (NK) cells in the culture was excluded by the finding that the cytotoxic capability of sensitized effector cells was greatly diminished by treatment with anti-Thy 1.2 serum and complement. Serologic analysis with anti-H-2 alloantisera revealed that the YAC-b tumor line, but not the parental YAC lymphoma, carried H-2^b alloantigens. It is concluded that the 'unpredicted' cytotoxic responses here observed is a result of conventional antigen recognition and of specific sensitization to alien H-2^b components expressed on the YAC-b subline.*

INTRODUCTION

THE YAC lymphoma was established in 1962 in an A/Sn(H-2^a) mouse by Moloney virus [1] and has since then been serially propagated in the ascites form in syngeneic mice. In the course of our recent studies on *in vitro* generation of cytotoxic lymphocytes against *in vivo*-passaged murine lymphoma cells, we observed a strikingly different growth pattern of YAC tumor in some of our A mice. In contrast to the original YAC tumor, which usually killed the host within 2 weeks after implantation of 10^7 cells intraperitoneally, some of the mice survived for more than 4–6 weeks. Although these mice had large amounts of ascites, only a relatively small number (less than 2×10^8) of tumor cells could be recovered. On further analysis, these cells, in contrast to the original YAC tumor, grew in and killed C57BL/6 (H-2^b) recipients and generated *in vitro* cytotoxic lymphocytes reactive with H-2^b targets. This subline of YAC lymphomas was later designated YAC-b.

These unexpected properties of the naturally-selected YAC-b subline led us to investigate its antigenic properties, using serological and cell-mediated cytotoxic assays. The findings reported herein show that the YAC-b subline expresses determinants identical to or cross-reactive with H-2^b alloantigens.

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MATERIALS AND METHODS

Tumor cells

All the tumor cells were propagated *in vivo* in ascites form in the syngeneic host: EL4, a DMBA-induced lymphoma of C57BL/6 mice (H-2^b); RBL-5, a Rauscher virus-induced lymphoma of C57BL/6 mice (H-2^b); YAC, a Moloney virus-induced lymphoma of A mice (H-2^a); and a mineral oil-induced plasmacytoma B.1 of BALB/c mice (H-2^d).

MLC and MLTC

Cultures were carried out under conditions carefully determined for optimal generation of cytotoxic T cells [2]. Stimulator spleen and lymphoma cells (20×10^6/ml, 5 ml) were treated with mitomycin C (Sigma) at a final concentration of 30 μg/ml and 60 μg/ml respectively

for 30 min at 37°C and washed 3 times in RPMI 1640 medium. Cultures were set in triplicate in 50 ml tissue culture flasks (Falcon No. 3013) with 30×10^6 responder splenocytes and $3–6 \times 10^6$ stimulator cells in a total volume of 20 ml, or in 17 × 100 mm culture tubes (Falcon No. 2057) with 5×10^6 responders and 5×10^5 stimulators in 5 ml volume. The culture medium was RPMI 1640 supplemented with 10% heat-inactivated FCS (Biolab Laboratories, Jerusalem), 10 mM Hepes buffer, 5×10^{-5} M 2-mercaptoethanol (2-ME) and antibiotics. Cultures were incubated upright for 5–6 days in a humidified atmosphere of 7% CO_2 in air at 37°C. As controls, responder lymphocytes were cultivated alone. After incubation, cells were spun down, washed once, counted in 0.1% trypan blue and suspended in 10% FCS–Hepes–RPMI 1640 medium.

[^{51}Cr] *cytotoxicity assay*

Lymphoma cells and mitogen-induced blasts were used as target cells. Blasts were prepared by cultivating splenocytes (30×10^6 in 15 ml of 10% FCS–Hepes–2-ME–RPMI 1640) for 2–3 days with 2.5 μg/ml of concanavalin A (Miles-Yeda, 3 times crystallized), followed by centrifugation on Ficoll-Hypaque and 3 washes. Two to 5×10^6 blasts and leukemic cells in 0.5 ml were labeled with 200 μCi of [^{51}Cr]-sodium chromate for 45 min at 37°C, washed 3 times and suspended in 10% FCS–Hepes–RPMI medium. Cell mixtures consisting of 2×10^5 viable sensitized lymphocytes and 2×10^4 labeled target cells (in 0.4 ml) were placed in 10 × 70 mm plastic tubes in triplicate. As controls, targets were incubated with medium alone. The cells were centrifuged for 3 min at 250 *g* and incubated for 3 hr at 37°C in a CO_2 incubator. After incubation the tubes were agitated vigorously, 1 ml of cold medium was added, the cells were pelleted by a 10 min centrifugation at 500 *g* and the supernatants transferred to new tubes and counted in a gamma scintillation counter. Percent cytotoxicity was calculated as follows:

$$\% \text{ cytotoxicity} = \frac{\%[^{51}\text{Cr}] \text{ release in test group} - \%[^{51}\text{Cr}] \text{ release in control group}}{\text{total releasable counts} - \%[^{51}\text{Cr}] \text{ release in control group}}$$
(80–90% by freezing and thawing)

Competitive inhibition assay

Sensitized effector cells (2×10^5, in 0.2 ml) were incubated at 37°C for 15 min either alone or with 2,4 and 8×10^5 (in 0.1 ml) unlabeled normal or tumor cells (competitor cells). Subsequently, [^{51}Cr]-labeled target cells (2×10^4 in 0.1 ml) were added and the assay proceeded as described above.

Treatment with anti-Thy 1.2 and complement (C′)

Sensitized effector cells (1×10^7) were incubated for 30 min at 37°C with 0.5 ml of a monoclonal anti-Thy 1.2 serum (diluted 1/1000). Cells were then spun down, resuspended in 0.3 ml of fresh absorbed rabbit C′ (1 ml rabbit serum absorbed twice for 30 min at 4°C with a mixture of 1×10^8 C57BL/6 and A splenocytes) diluted 1/6–1/8, and incubated for an additional 45 min at 37°C. After incubation, effector cells were washed twice and tested in the [^{51}Cr] release assay. Controls comprised of cells incubated with either antiserum or C′ alone. Under the conditions employed, no significant reduction (less than 10%) in NK activity against sensitive lymphoma target cells was noted when freshly obtained splenocytes (BALB/c, C57BL/6, A) were pretreated with anti-Thy 1.2 plus C′.

Treatment with anti-H-2 alloantisera and C′

Antisera were produced by immunizing H-2-incompatible allogeneic mice with pooled spleen, kidney and liver suspensions (1 donor for 20 mice), and inoculated subcutaneously at biweekly intervals during 10–16 consecutive weeks. The mice were bled 7–10 days after last injection and the sera were heat-inactivated. In the C′-dependent microcytotoxicity test, 80–100% of spleen cells were lysed in the presence of 1/256–1/1024 dilutions of the respective alloantisera. For detection of H-2 alloantigens on splenocytes and lymphoma cells, the microcytotoxicity test was employed as follows. Ficoll-Hypaque-separated splenocytes (> 92% viable) and lymphoma cells (5×10^4 in 25 μl) were incubated in 96-well U-shaped microtitration plates for 30 min at room temperature with alloantisera (diluted 1/16, in 25 μl); subsequently, absorbed rabbit C′ (diluted 1/8, in 50 μl) was added and the cells were incubated for additional 45 min at 37°C. As controls, cells were incubated with either alloantiserum or C′ alone. The number of dead cells was determined by the trypan blue dye exclusion technique.

RESULTS AND DISCUSSION

Table 1 shows the results of the cytotoxic

*Table 1. 'Unpredicted' cytotoxicity generated in MLC and MLTC**

Group No.	Sensitization system Responders	Stimulators	Percentage cytotoxicity of: (E/T = 10/1)† BALB/c B.1 Plasma-cytoma	C57BL/6 Con A-blasts	C57BL/6 EL4 lymphoma‡	A Con A-blasts	A YAC-b lymphoma	A YAC lymphoma
1	BALB/c	None	0	4	5	3	6	4
2	BALB/c	C57Bl/6 splenocytes	0	70	92	7	72	16
3	BALB/c	EL4	ND§	85	88	4	65	11
4	BALB/c	A splenocytes	1	16	15	61	66	75
5	BALB/c	YAC-b	ND	77	68	55	80	71
6	BALB/c	YAC	4	19	17	62	77	83
7	A	None	2	2	5	2	5	2
8	A	C57BL/6 splenocytes	0	69	86	2	82	11
9	A	EL4	ND	78	84	3	76	10
10	A	BALB/C splenocytes	43	29	37	4	39	33
11	A	YAC-b	ND	32	58	1	36	17
12	A	YAC	ND	5	6	0	19	15
13	C57BL/6	None	0	1	1	0	2	0
14	C57BL/6	A splenocytes	32	1	1	47	52	61
15	C57BL/6	BALB/C splenocytes	28	1	1	52	60	49

*Sensitization was carried for 5–6 days at R/S ratios of 5/1 or 10/1.

†Values represent means of 3–7 separate experiments in each group. Underlined numbers indicate 'unpredicted' cytotoxic reactivity.

‡Similar results were obtained with RBL-5 lymphoma as target cells.

§Not done.

response generated in MLC and MLTC in various combinations of responder and stimulator cells. In 10 combinations of alloreactivity and in two syngeneic MLTC systems (groups 11, 12), cytotoxicity against the sensitizing antigens was detected. With Con A-induced blasts, B.1 plasmacytoma and the EL4, RBL-5 and YAC lymphoma target cells, the reactivities were as expected according to the alloantigens expressed. In certain MLC combinations, such as BALB/c anti-A (group 4) and A anti-BALB/c (group 10), a certain degree of cross-reactivity with C57BL/6 target cells occurred, probably due to shared H-2 public determinants in these three strains of mice. Against the YAC-b subline, however, all mixed cultures had cytotoxicity. Killing occurred with high efficiency, even if the results with Con A-induced A strain blasts or the parental YAC tumor indicated only slight or no cross-reactivity and, more important, even with A responder cells syngeneic to the targets (groups 8–11). Moreover, allogeneic and syngeneic sensitization against YAC-b cells (groups 5 and 11 respectively), but not against YAC or A splenocytes, elicited a strong cytotoxic effect to normal and leukemic C57BL/6 targets. Thus, 'unpredicted' cytotoxic response was observed with the YAC-b lymphoma variant of strain A mice when employed either as sensitizer or as target, in contrast to the predicted reactivity obtained with the original YAC lymphoma line and splenocytes of strain A mice.

The 'unpredicted' behavior of YAC-b cells was demonstrated further in competitive inhibition assays (Fig. 1), where effectors induced by C57BL/6 stimulator cells were inhibited to the same extent by unlabeled YAC-b and EL4 competitor cells, using both YAC-b and EL4 as targets. In contrast, YAC cells exhibited only marginal competition comparable to that of Con A blasts of strain A origin.

The possibility that the 'unpredicted' cytotoxic reactivity detected with YAC-b targets was due to activation of killer cells against C-type virus determinants can be excluded, because in that case RBL-5 and EL4 cells, which express virus-determined antigens cross-reactive with YAC cells [3, 4], would have been killed also. Neither can this reactivity be attributed to the action of classic NK effectors, which may be induced in culture [5, 6], since cytotoxicity to

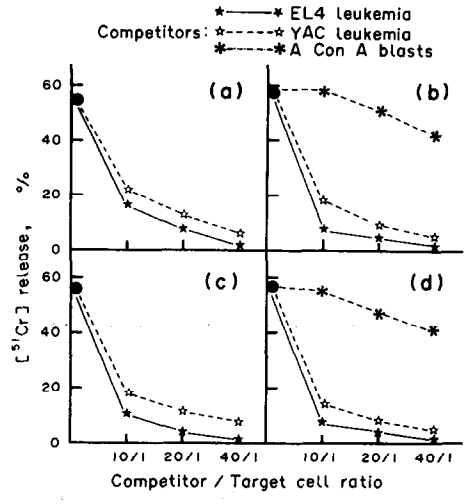

Fig. 1. Competitive inhibition assay. In (a) and (b), BALB/c splenocytes were sensitized in MLC against C57BL/6 splenocytes; in (c) and (d), A splenocytes were sensitized against C57BL/6 splenocytes. [51Cr]-labeled target cells were EL4 (a and c) and YAC-b (b and d) lymphomas; competitor cells were unlabeled EL4, YAC-b and Con A-induced blasts of A splenocytes. The parental YAC line exhibited the same pattern of inhibition as strain A blasts.

YAC-b cells was highly sensitive to anti-Thy 1.2 serum plus C′ (Table 2) under conditions where freshly obtained NK effector cells were not affected. Furthermore, other studies revealed that the YAC-b line was relatively resistant to NK cells. The serological analysis with the use of alloantisera and C′, showing that the YAC-b variant, but not the parental YAC nor A splenocytes, strongly reacts with A anti-C57BL/6 serum (Table 3), leads us to conclude that the 'unpredicted' cytotoxicity observed with the YAC-b variant as sensitizer and as target results from specific stimulation by antigens identical to or cross-reactive with H-2^b antigens present on these tumor cells but not on the parental YAC tumor.

The shift in the antigenic make-up of some tumor cells may occur immediately after cell transformation or in the course of *in vitro* and *in vivo* passages of tumor cells for prolonged periods of time (our YAC has been maintained *in vivo* for approximately 20 years). The appearance of the additional ('alien', 'inappropriate') H-2 markers on tumor cells [7–11] may not necessarily be the result of repressed gene activation, but rather it can reflect increase in quantity or exposure of (inaccessible) components already present in the parent normal cell membrane [12]. It must also be pointed out that in addition to the H-2^a/H-2^b subline of YAC tumor described here, we have recently obtained from our leukemic mice another subline deficient in both H-2^a and H-2^b determinants and which grows progressively in A, BALB/c and C57BL/6 mice.

It seems unlikely that the antigenic drift described in this work—the acquisition of H-2^b antigens—resulted from mixing up H-2^a and H-2^b tumor lines during *in vivo* passages of our lymphomas. Over the last 3 years, we and another independent group at our institute in Jerusalem have repeatedly observed this antigenic change following inoculation of our frozen stock of YAC tumor into A mice. It should be emphasized that no (A × C57BL/6)F_1 hybrids have been carried in our laboratories.

It is still not clear as to the nature of the selection force in our A mice responsible for this change. The ability of the H-2^a/H-2^b variant to grow progressively in A mice in spite of the presence of the allo-determinants may

*Table 2. Reduction of cytotoxic activity generated in MLC by treatment of sensitized effector cells with anti-Thy 1.2 serum + C′**

Sensitization system		Treatment of	Percentage cytotoxicity against:†	
Responders	Stimulators	effector cells	EL4	YAC-b
BALB/c	C57BL/6	Anti-Thy 1.2	84	89
		C′	82	90
		Anti-Thy 1.2 + C′	32	43
A	C57BL/6	Anti-Thy 1.2	86	90
		C′	84	91
		Anti-Thy 1.2 + C′	21	22
A	BALB/C	Anti-Thy 1.2	42	71
		C′	38	70
		Anti-Thy 1.2 + C′	10	14

*MLC were carried for 5–6 days; sensitized cells were treated as described in Materials and Methods and tested against [51Cr]-labeled lymphoma cells at E/T = 10/1.
†The values are means of 3 separate experiments.

Table 3. Detection of H-2^b alloantigens on YAC-b lymphoma cells by anti-H-2^b alloantiserum and complement

Alloantiserum	Percentage of killed cells*				
	A splenocytes†	YAC-b lymphoma	YAC lymphoma	C57BL/6 splenocytes†	EL4 lymphoma
A anti-C57BL/6	10	73	14	98	98
C57BL/6 anti-A	94	80	93	8	8
BALB/c anti-C57BL/6	92	91	19	97	93
C' alone	7	10	8	7	5

*The values are means of 4 separate experiments.
†Before testing, splenocytes were separated on a Ficoll-Hypaque layer.

result from inability of the alien determinants to elicit an effective rejection response. Another possibility is that the *in vivo*-carried YAC tumor is a potent inducer of suppressor cells [13] that may counteract the allogeneic response.

In conclusion, it would appear from these experiments that cross-reactive or 'unexpected' cytotoxic reactivities that have been exhibited in many studies employing tumor cells as sensitizers or as targets may be due, at times, to the expression of alien histocompatibility antigens that may appear spontaneously on long-transplanted or *in vitro*-carried tumor cells.

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