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## Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy

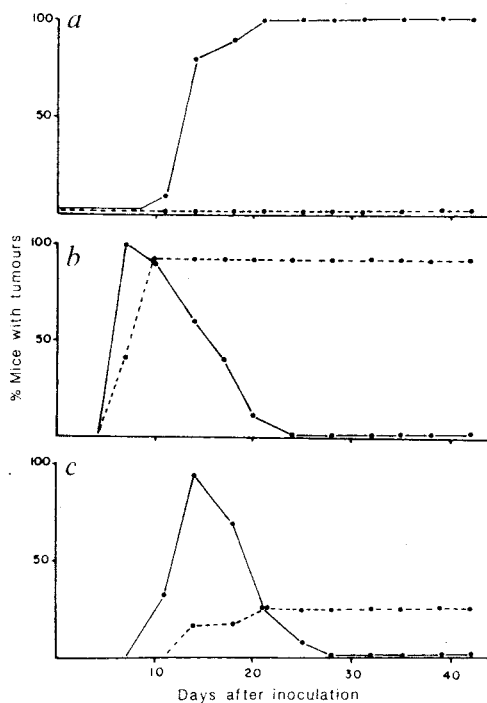
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Metazoan organisms may discriminate between self and non-self not only by the presence of foreign antigens but also by the absence of normal self markers<sup>1</sup>. Mammalian adaptive immune responses use the first strategy, with the additional requirement that foreign antigens are recognized in the context of self-major histocompatibility complex (MHC) products at the cell surface<sup>2</sup>. Aberrant cells which fail to express MHC products adequately can therefore avoid detection<sup>2–4</sup>. A more primitive but complementary defence system, eliminating such cells on the basis of absent self-markers, is suggested by a re-interpretation<sup>5,6</sup> of phenomena associated with metastasis and natural resistance. We now show that murine lymphoma cells selected for loss of H-2 expression are less malignant after low-dose inoculation in syngeneic hosts than are wild-type cells, and that the rejection of such cells is non-adaptive. On the basis of our data, we suggest that natural killer cells are effector cells in a defence system geared to detect the deleted or reduced expression of self-MHC.

More specifically, the hypothesis<sup>5,6</sup> stated that self-MHC genes influence the expression or recognition of inhibitor signals in interactions between potential target cells and natural effector cells. Our experimental approach was to test the prediction that selection for loss of H-2 in tumour lines should be accompanied by an increased sensitivity to natural resistance *in vitro* and *in vivo*. Three independent anti-H-2-resistant sublines from two lymphomas were selected. Such variant cells not only were able to escape adaptive alloreactivity *in vitro* and *in vivo*, but, as predicted by our hypothesis, had also become unable to grow

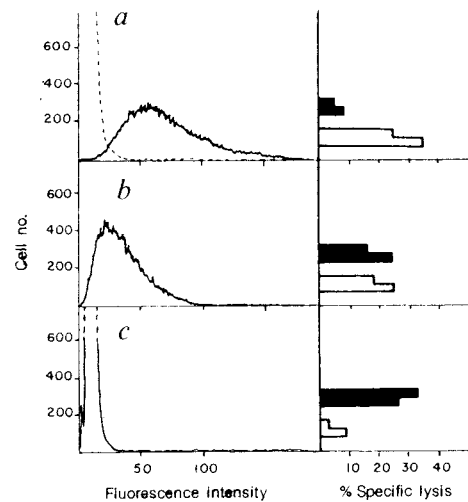


**Fig. 1** Proportion of syngeneic and allogeneic mice with palpable tumours at different time points after subcutaneous injection of RMA (RBL-5, mutagenized but not selected; solid line) and RMA H-2 sel. (mutagenized, selected, H-2-resistant subline; dashed line). *a*,  $10^4$  cells in syngeneic C57BL mice; summary of two experiments with a total of 12 mice for each tumour. *b*,  $10^6$  cells in allogeneic A.BY mice; summary of four experiments with a total of 18–21 mice for each tumour. *c*,  $10^3$  cells in allogeneic A.BY mice; summary of four experiments with a total of 18–21 mice for each tumour.

out from small inocula in the normal syngeneic host, and switched from the natural killer (NK)-resistant to the NK-sensitive phenotype as a consequence of the selection against H-2 expression.

We used the RBL-5 lymphoma (H-2<sup>b</sup>) for these studies because it readily expresses H-2 and is highly malignant in the normal syngeneic host. In contrast to two other C57BL-derived lymphomas, the number of tumour takes after a threshold inoculum was not significantly increased in NK-cell-defective mice<sup>7</sup>. This lymphoma is therefore probably regarded as close to 'normal self' by natural resistance mechanisms in the syngeneic or H-2<sup>b/b</sup>-compatible hosts. In contrast, the subline that had been selected for loss of H-2 expression (RMA H-2 sel) failed to grow in such mice, even after a 10-fold increase in the dose required for 100% takes of the wild-type tumour (Table 1). The subline had been derived after mutagenization and repeated treatment with alloantiserum and complement. It was completely resistant to this treatment, due to a selective loss or reduction in expression of MHC class I gene products (Table 1) and  $\beta_2$ -microglobulin; it did not stain significantly above normal control serum with alloantibodies on analysis by fluorescence-activated cell sorter (FACS) (Fig. 1), while binding and complement-dependent killing with anti-Thy 1.2 antibodies were unaffected (Table 1). The wild-type and the selected line were both negative for monoclonal antibodies against TL and I-A<sup>b</sup> antigens (not shown).

In view of the proposed hypothesis, it was important first to study natural resistance in syngeneic (C57BL) or at least semi-syngeneic (A.BY  $\times$  C57BL) H-2<sup>b/b</sup> homozygous hosts, in order to avoid combinations producing strong T-cell reactivity. In an attempt to obtain the latter situation in subsequent studies,



**Fig. 2** H-2 expression, susceptibility to allospecific cytotoxic T lymphocytes (CTL), and poly-I:C-induced NK cells of RBL-5 sublines established after mutagenization, before selection (= RMA, *a*), after two selections with alloantiserum + complement (*b*) and after the completed series of five selections (= RMA H-2 sel., *c*). Left-hand panels show H-2<sup>b</sup> expression by FACS analysis after indirect staining with hyperimmune A/Sn anti-A.BY alloantiserum (1/20) and fluorescent conjugated rabbit anti-mouse immunoglobulin (Dakopatts). The fluorescence profiles were determined on a FACS IV using a laser light output of 200 mV, photomultiplier at 450 V and fluorescence gain  $\times 2$ . Nonspecific background staining, estimated by using the reciprocal antiserum A.BY anti-A/Sn (anti-H-2<sup>a</sup>) as the first antibody, was similar for the three lines and is shown here for the nonselected line only (dashed curve, *a*). Right-hand panels show the sensitivity of the corresponding cell lines to anti-H-2<sup>b</sup> cytotoxic T cells derived from A/Sn anti-A.BY mixed leukocyte cultures, and tested at an effector/target ratio of 6/1 (open bars, with the upper and lower part corresponding to the same two independent tests for all cell lines) and splenocytes from H-2-syngeneic C57BL or (A.BY  $\times$  C57BL)F<sub>1</sub> mice injected with poly-I:C (150  $\mu$ g intraperitoneally 18 h before test), tested at an effector/target ratio of 100/1 (solid bars, with the upper and lower part corresponding to the same two independent tests for each of the cell lines). The mixed lymphocyte cultures and the 4–6-h <sup>51</sup>Cr-release assays were set up as described previously<sup>39</sup> and all three lines were tested in parallel in each of the experiments.

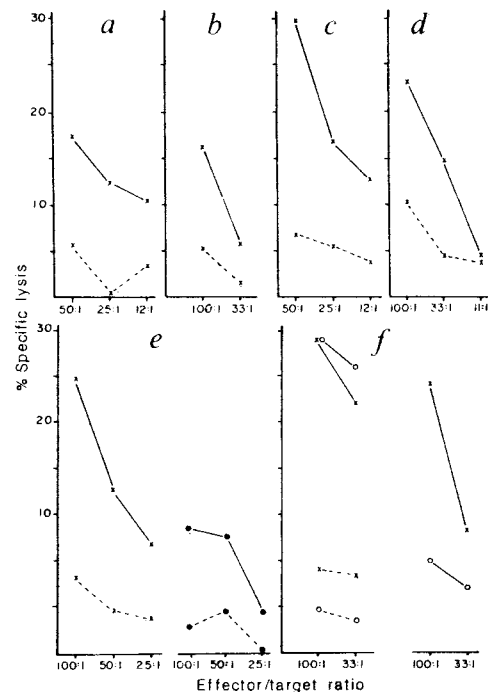
high-dose inocula were given to inbred A.BY mice (rather than outcrossed to C56BL), presenting a minor histocompatibility barrier to the RBL-5 lymphoma<sup>8</sup>. The pattern of progressive tumour growth then changed to the opposite: H-2-variant lymphoma cells grew progressively once they had formed a tumour, whereas all wild-type tumours were rejected after temporary growth (Fig. 1). High-dose challenge ( $1 \times 10^6$  cells) of preimmunized syngeneic C57BL mice gave a similar picture: complete protection was seen with the wild-type line, while H-2-resistant cells failed to immunize against themselves (Table 1). These results and observations on tumour outgrowth in pre-irradiated hosts (Table 1) indicated that the reduced tumorigenicity of H-2-lacking variant cells was not due to an intrinsic inability to grow *in vivo*, but rather to an active host defence, dependent on radiosensitive (precursor) cells, but independent of tumour immunogenicity in the classical sense. It was important to establish that this was the case, in view of reports that low malignant mutant or transfectant tumour lines can acquire the ability to elicit strong adaptive T-cell responses<sup>9–12</sup>. Indeed, the variants seemed to escape T-cell recognition *in vivo*, probably because minor histocompatibility antigens and tumour antigens are recognized only in the context of self-MHC products<sup>2,13</sup> and thus fulfilled one of the criteria for action by the postulated defence system. The variant cells were also able to escape anti-H-2<sup>b</sup>

Table 1 Summary of experimental results

Sublines			Serology								Tumour growth <i>in vivo</i>					
Original line	Mutagenization series selection	Designation	A/Sn → A.BY alloantisera		20-8-4S K <sup>b</sup> D <sup>b</sup> mab		β <sub>2</sub> M rabbit mab	β <sub>2</sub> M rabbit mab	F7D5 Thy 1.2 mab		Untreated				Pre-immunized	Pre-irradiated
			CX	FACS	CX	FACS	CX	FACS	CX	FACS	10 <sup>3</sup>	10 <sup>4</sup>	10 <sup>5</sup>	10 <sup>6</sup>	10 <sup>6</sup>	10 <sup>4</sup>
RBL-5	A—none	RMA	++	++	++	++	+	++	++	++	7/7	16/16	10/10	9/9	1/9	9/9
RBL-5	A—A/Sn → A.BY	RMA H-2 sel.	—	—	—	(±)	—	(±)	++	++	0/9	0/14	NT	5/5	5/5	4/4
RBL-5	B—A/Sn → A.BY	RMB H-2 sel.	—	—	—	(±)	—	(±)	++	++	1/14	0/16	1/12	4/5	5/5	5/5
EL-4	A—none	EL-4MA	++	++	++	++	NT	NT	NT	NT	3/10	6/10	7/10	NT	NT	NT
EL-4	A—A/Sn → A.BY	EL-4MA H-2 sel.	—	(±)	—	(±)	NT	NT	NT	NT	0/10	1/13	1/14	NT	NT	NT

**Sublines:** RBL-5 is a Rauscher virus-induced lymphoma and EL-4 a benzyrene-induced leukaemia, both originating in the C57BL strain. The cells, maintained in RPMI with 10% fetal calf serum and penicillin-streptomycin, were mutagenized with ethanemethanesulphonate (EMS, Sigma), 200 μg ml<sup>-1</sup> for 24 h. After washes, the cells were allowed to recover for 1 week and then treated with alloantisera (A/Sn → A.BY diluted 1:20, 10<sup>7</sup> cells ml<sup>-1</sup>) followed by rabbit complement (Pel Freez, 1/10) for 1–4 h. The cells were cultured without any attempts to remove the dead cells, and the survivors (<0.25%) were expanded. The treatment was repeated at least four times. **Serology:** The A/Sn → A.BY (anti-H-2<sup>b</sup>) serum was produced in our animal colony at the Karolinska Institute. The monoclonal antibody (mab) 20-8-4S (ref. 38) was obtained from the American Type Culture Collection. The anti-β<sub>2</sub>-microglobulin (β<sub>2</sub>M) reagents were kindly sent by Drs Shen and Boyse (Sloan Kettering Memorial Institute, New York) (mab) and Dr Tanigaki (Roswell Park Memorial Institute, Buffalo) (rabbit antiserum). The F7D5 reagent was a gift from Dr E. Clark (Genetic Systems, Seattle). CX, complement-dependent cytotoxicity in a two-step assay evaluated by trypan blue exclusion. ++, 95–100% of cells killed, reciprocal titre no less than 100. +, 70–95% of cells killed, reciprocal titre 10–50; —, below background killing with complement alone. FACS, indirect fluorescence, after a two-step staining procedure using a FACS IV (Becton & Dickinson) with a laser output of 200 mV, photomultiplier at 600 V and a constant fluorescence gain for each antibody. The settings used gave a background median fluorescent intensity (MFI) below 10 on a linear scale (using normal mouse of rabbit serum, C57BL antisera to other H-2 haplotypes or T-lymphoma supernatant instead of the specific antibody in the first incubation). ++, MFI > 100 under the same conditions; +, MFI 20–100; (±) MFI, 5–19; —, below background. Secondary reagents: fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulin or swine anti-rabbit immunoglobulin (Dakopatts). The cells were incubated at 10<sup>7</sup> ml<sup>-1</sup>, 30 min on ice in both steps. **Tumour growth *in vivo*:** All tests were with ascites tumours, serially passaged in 400R-pre-irradiated mice to avoid immunoselection *in vivo*. Different numbers of tumour cells were injected subcutaneously to groups of three to six H-2-syngeneic hosts, either C57BL or (A.BY × C57BL)<sub>F</sub><sub>1</sub> for all tumours in each experiment. The two types of mice gave consistent results, and the number of mice with tumour takes (out of totally injected) have therefore been pooled from several different experiments, each one including wild-type and variant cells injected to parallel groups. Two experiments with 10<sup>5</sup> cells included parallel groups of preirradiated mice (400R, 24 h before tumour challenge). The data with 10<sup>6</sup> injected cells represent two experiments where half of the mice had been preimmunized with the challenging tumour (three weekly injections of 2 × 10<sup>7</sup> 10,000 R irradiated RMA or RMA H-2 sel. cells). NT, not tested.

**Fig. 3** Susceptibility of wild-type and H-2-selected sublines to killing by spleen cells from poly-I:C-treated (150 μg per mouse intraperitoneally 18 h before injection) or untreated mice. *a, b*, Two independent experiments with effector cells from poly-I:C-treated C57BL mice tested against EL-4MA (dashed line) and EL-4MA H-2 sel (solid line). *c, d*, Two independent experiments with effector cells from poly-I:C-treated C57BL (*c*) and (A.BY × C57BL)<sub>F</sub><sub>1</sub> (*d*) mice tested against RBL-5 wild-type (dashed line) and RMB H-2 sel. (solid line). *e*, Nylon wool column-passed<sup>35</sup> spleen cells from normal C57BL (×) and A.BY (○) mice tested against RMA (dashed line) and RMA H-2 sel. (solid line) in a 16-h <sup>51</sup>Cr release assay. *f*, Left, effector cells from poly-I:C-treated (A.BY × C57BL)<sub>F</sub><sub>1</sub> mice tested against RMA (dashed lines) and RMA H-2 sel. (solid lines), with (○) and without (×) pretreatment of targets with β-interferon (Stratech Scientific Ltd, 3.6 × 10<sup>7</sup> immunoreactive units per mg protein, cat. no. 83001), 5 × 10<sup>3</sup> immunoreactive units ml<sup>-1</sup>, 18 h; *f*, right, effector cells from poly-I:C-treated A/Sn mice tested against YAC-1 cells with (○) and without (×) pretreatment of targets with β-interferon as described above. Median H-2 fluorescence intensity by FACS analysis: RMA, 105, β-interferon-treated RMA, 180; RMA H-2 sel. <15 before as well as after β-interferon treatment; YAC-1, 28; β-interferon-treated YAC-1, 71 (staining procedure as in Fig. 2, except that A.BY anti-A/Sn H-2<sup>a</sup> serum was used as the first antibody with YAC-1 cells; background was 6–11 for all lines. All cytotoxicity tests were performed with the <sup>51</sup>Cr-release assay, as described previously<sup>39,40</sup>, with an incubation time of 4–6 h, except in *e* (16 h).



cytotoxic lymphocytes *in vitro* (Fig. 2) as a result of the selection. However, at the same time, they had switched from the NK-resistant to the NK-sensitive phenotype (Fig. 2).

Although the *in vivo* results presented here do not prove definitively that the natural resistance was mediated by NK cells, the non-adaptive nature of the rejection and the results on irradiated hosts are consistent with this interpretation. The dose of 400R reduces splenic cellularity by 95%; even if some functional NK cells remain, the total activity in terms of lytic units per animal is reduced within 24 h, and falls to zero within the following weeks, that is, the period between tumour injection and outgrowth in our experiments<sup>14</sup>. This enhanced sensitivity to natural resistance *in vitro* and *in vivo* was observed with an independently derived H-2-resistant RBL-5 subline, as well as

with an EL-4 subline selected in a similar fashion (Table 1, Fig. 3). The general lysability of the wild-type control line was also confirmed in the case of EL-4, which was efficiently killed (>50% lysis at effector target/ratio 5:1) by anti-H-2-specific, mixed lymphocyte culture-derived effector cells (data not shown).

According to the proposed model, the tumour cells would be unable to inhibit triggering of NK lysis after complete or partial loss of self H-2<sup>5,6</sup>. This would be consistent with several observations on NK cells, including high sensitivity of *in vitro*-grown YAC-1 lymphoma cells, immature thymocytes and teratocarcinoma cells, all of which have low or defective MHC expression<sup>15,16</sup>. The corresponding cells show reduced sensitivity when H-2 is induced (by differentiation<sup>17,30</sup>, interferon<sup>19</sup> or

growth *in vivo*<sup>2</sup>). NK-cell-mediated rejection of H-2-disparate bone marrow and lymphoid grafts, including the F<sub>1</sub> hybrid resistance phenomenon<sup>18,21,22</sup> and early elimination of allogeneic lymphocytes<sup>23</sup>, are other examples that could be reinterpreted to reflect recognition of incomplete self-H-2 expression: allogeneic grafts lack H-2 genes of the recipient, and parental grafts fail to match one haplotype, that is, they lack 50% or more of the total 'self'-H-2 molecules expressed in the F<sub>1</sub> recipient environment. This predicts that the relationship between reduced self expression and NK susceptibility should be quantitative rather than dependent on complete deletion of H-2 products, as is indeed supported by findings with a subline of RBL-5 derived after the two first selections with anti-H-2 + complement. These cells had an average loss on H-2 fluorescence corresponding to more than 50% of the total H-2 expression in the original line, and a clear increase in NK sensitivity (Fig. 2).

The hypothesis would also explain why it is difficult to select H-2<sup>-</sup> variants *in vivo*<sup>24</sup>, and predict a selection for increased H-2 expression during tumour progression. The correlation between metastatic potential and increased expression of all<sup>25</sup> or certain (D-end encoded<sup>26,27</sup>) MHC class I alleles observed for several experimental tumours could therefore be reinterpreted as selection of phenotypes able to escape NK cells. However, preferential outgrowth of cells with reduced or deleted expression of certain MHC alleles (often H-2K-end encoded) has been observed after large-dose inoculation ( $\geq 10^5$  cells) of some virally induced, strongly antigenic tumours<sup>12,28,29</sup>. T cells presumably represented the major surveillance mechanism in those systems, as they would in the present experiments, where H-2-deficient sublines grew out preferentially after transplantation of large-dose inocula to preimmunized C57BL or histoincompatible A.BY mice (Table 1, Fig. 1).

Burnet pointed out the fundamental difference between strategies for self-non-self-discrimination in invertebrates and mammals<sup>1</sup>. In the colonial tunicate *Botryllus*, rejection of allogeneic cells as well as control against self-fertilization is thought to operate by a mechanism scanning for the presence or absence of self-markers, encoded by a single locus with considerable polymorphism<sup>1,31</sup>. Such a system may also have been fixed in mammals, despite the development of adaptive immunity. The selective pressures would have required not only a back-up system eliminating aberrant (MHC loss) cells escaping detection by T lymphocytes, but also a rapid first-line defence with a certain selectivity. Examples exist of lytic as well as transforming viruses that cause a profound but selective reduction of H-2 expression in infected cells<sup>30,32</sup>. The latter would be eliminated by the proposed defence mechanism, while sparing of normal cells in the tissues would be guaranteed by an increase in their H-2 expression due to interferon stimulation early in immune responses and NK-cell activation. Interferons do reduce the NK susceptibility of many targets<sup>33</sup>; the mechanisms involved are unknown, but may be related to MHC gene regulation as suggested by observations on variant cells in this (Fig. 3f) and another study<sup>41</sup>.

The present findings may also be relevant to previous speculations by Snell<sup>34</sup> and Dausset<sup>35</sup> concerning the primordial functions of MHC genes, that is, functions that would not be related to T-cell restriction. The possibility of a common regulation of H-2 expression, NK-cell-resistant phenotype and malignancy properties in lymphoma cells is also very interesting in relation to recent advances in the research on regulation of MHC class I genes, particularly in tumour cells<sup>36,37</sup>.

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