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Decreased Sensitivity of Immunoselected and NK-selected YAC Lymphoma Sublines to Hybrid Resistance *In Vivo**

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Abstract—*Two sublines of the Moloney lymphoma YAC, selected by alternating* in vitro *exposure to anti-MCSA + complement and* in vivo *passage in preimmunized hosts, had a decreased or undetectable expression of MCSA. These 'immunoselected' sublines were compared with the original YAC line with regard to their ability to grow in a panel of semisyngeneic F_1 hybrids. Natural hybrid resistance to YAC, previously found to be mediated by NK cells, affected the immunoselected sublines to a much smaller extent. This was further corroborated by the fact that the same sublines showed a decreased sensitivity to the* in vitro *lysis by NK cells from the same hybrid genotypes. Another set of YAC variants were produced by repeated* in vitro *exposure to NK cells and intermittent passage in highly NK-active F_1 hosts. These 'NK-selected' sublines showed a permanently decreased sensitivity to NK lysis after* 8–10 *selections. When compared for* in vivo *growth with the parental YAC-1 tissue culture line in a spectrum of relatively resistant F_1 hybrids, they had an increased frequency of takes. This is in line with recent findings which show a relationship between the target site for natural antibodies and anti-MCSA on the one hand, and between the natural antibody-binding site and the NK target site on the other.*

INTRODUCTION

HYBRID resistance to parental tumors was first described by Snell [1] and later confirmed by others [2, 3]. Its mechanism and genetic control are still not completely known. In our previous studies we have compared the resistance pattern in a spectrum of F_1 hybrid genotypes against tumors of various histological types induced by different agents in several inbred strains of mice [4, 5]. There were certain consistencies in the resistance pattern, particularly in relation to the lymphomas. Some F_1 hybrids were resistant against all 9 C57BL-derived lymphomas tested, induced by different viruses and chemical carcinogens, whereas others were not resistant. Tests in H-2 congenic and in backcross mice showed frequent association between resistance and certain H-2 haplotypes. Non-H-2 linked genes were also involved, however.

While the inducing agent appeared to have no influence on the hybrid resistance pattern, there appeared to be a certain relationship between the histological type and the resistance pattern. Carcinomas and sarcomas showed genetic patterns that differed from the lymphomas as a rule [4, 5].

The mechanism of hybrid resistance has only been studied in a few systems. For some lymphomas, a close parallellism has been found between the genetic control of *in vivo* resistance and natural killer (NK) cell activity *in vitro* [6–9]. Also, NK-deficient beige mutant mice [10] were less resistant to small inocula of transplantable NK-sensitive lymphomas than their heterozygous littermate controls [11, 12]. For the YAC lymphoma, the target cell of the present study, hybrid resistance was entirely thymus-independent [13]. We have also shown that hybrid resistance against four H-2^b lymphomas was equally thymus-independent [14]. In contrast, the strong H-2^b-linked hybrid

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resistance against the NK-insensitive, strain A.SW-derived Moloney lymphoma YWA was largely mediated by *T* cells [15].

In the present study we have approached the mechanism of hybrid resistance from another angle. In the course of our earlier work we selected immunoresistant sublines from the original YAC lymphoma line by repeated *in vitro* exposure to complement-dependent cytotoxicity of serum from semisyngeneic mice immunized with irradiated YAC cells, designated as anti-MCSA (Moloney virus Cell Surface Antigen) antibody, followed by intermittent passages in pre-immunized hosts [16]. The derived YAC–IR subline was completely resistant to anti-MCSA serum + complement, but remained sensitive to anti H-2^a antibodies. Its MCSA concentration was only one tenth compared to YAC, whereas its H-2 expression was unaffected [17]. YAC462-IR is an independently selected subline that showed a similar reduction of MCSA antigen concentration and resistance to the corresponding antibodies as YAC–IR [18].

The purpose of our present study was to test whether the two immunoselected YAC sublines have also become refractory to the natural *in vivo* resistance of non-immunized semisyngenic F_1 hosts. The results were expected to reflect on the relationship between the target structures involved in natural and immune resistance against the same lymphoma.

As a suitable counterpart to the immunoselected variants we have also included a second series of YAC-sublines, obtained by repeated *in vitro* exposure to cytotoxic NK cells and intermittent *in vivo* passage in high NK-reactive F_1 hybrids [19]. These variants were relatively insensitive to NK-lysis *in vitro*. They were included to compare the effects of immune vs natural selection and to test whether resistance to NK-lysis *in vitro* is accompanied by a corresponding decrease of natural hybrid resistance *in vivo*.

MATERIALS AND METHODS

Mice

All homozygous mice were derived from our single line brother–sister mated inbred nucleus. F_1-hybrid matings included both reciprocal crosses without discrimination.

Tumors

YAC is a Moloney virus-induced lymphoma of strain A/Sn origin [20]. YAC–IR and YAC462–IR are independently selected immunoresistant variants of YAC described in detail elsewhere [16, 18]. Briefly, they were exposed to anti-Moloney serum + complement *in vitro* prior to each passage in Moloney virus-immunized mice. This double selection was repeated 8 times. All three lines were kept by serial transplantation in the syngeneic host in the ascites form. YAC–IR was karyotyped; it had the same near-diploid, stemline number of chromosomes as the original line, with trisomy of chromosome 15 [21].

YAC-1 is a permanent tissue culture line derived from YAC. YAC–ACK8, YAC–ACC10 and YAC–ACG9 have been derived from YAC-1 by alternating *in vitro* exposure to highly active NK cells of A × CBA or A × C3H origin, followed by *in vivo* passage in the F_1-hybrids [19]. These variants showed a stable decrease in their sensitivity to the *in vitro* lytic effect of NK cells after 8–10 selections. They were maintained *in vitro*. The karyotype of these sublines was indistinguishable from the original YAC line (T. Dalianis, personal communication).

Prior to their use for the present inoculation experiments, the three NK-resistant variants were inoculated i.p. in parallel with the parental YAC-1 line and passaged for two generations in the syngeneic strain of origin.

Inoculation tests

Syngeneic strain A/Sn and semisyngeneic F_1-hybrid mice were inoculated with 10^3 cells subcutaneously. The cell dose was chosen to give more than 50 but less than 100% progressively growing tumors in syngeneic mice. Tumor growth was followed by weekly inspection and palpation. The cumulative incidence of tumors was recorded. The mice were kept under observation for at least 2 months. All mice that developed tumors after the inoculation of YAC, YAC–IR or YAC462–IR died from progressive tumor growth. The tissue culture lines regressed occasionally, however, when inoculated subcutaneously, even after two passages in the ascitic form.

NK assay

The spleens of 5 to 8-week-old mice were tested in a [^{51}Cr] release assay against the tissue culture lines of YAC-1, YAC–ACK8, YAC–ACC10 and YAC–ACG9, and against the *in vivo*-maintained ascites lines YAC, YAC–IR and YAC462–IR. Three spleens were pooled in each experiment and the cell concentration was adjusted to 10^7 per ml; 100 μl was added to each well. Subsequently, 100 μl of the target cell suspension was added in a concentration of

10^5 cells per ml, giving an effector : target cell ratio of 100:1. The test medium was RPMI 1640, supplemented with 10% newborn calf serum and penicillin–streptomycin. After 4 hr of incubation, 100 μl supernatant was harvested and counted in a gamma spectrometer.

The percentage cytotoxicity was calculated according to the following formula:

$$\text{percentage lysis} = \frac{\text{cpm in test} - \text{cpm in medium alone}}{\text{total cpm} - \text{cpm in medium alone}} \times 100.$$

Cytotoxic T-cell assay

Spleen cells 15×10^6 of 5-week-old C57BL mice were cocultivated with 7.5×10^6 2000 rad-irradiated strain A/Sn spleen cells in a total volume of 15 ml. The RPMI medium was used supplemented with 10% fetal calf serum, penicillin–streptomycin, 10^{-5} M 2-mercaptoethanol and HEPES. On day 6 the cultured cells were tested for cytotoxicity against the ascites lines YAC, YAC–IR and YAC462–IR, as in the NK-assay.

MCSA expression

Anti-Moloney serum was produced by inoculating 6000 rad-irradiated YAC cells into semisyngeneic A × C57BL recipients. After 5 immunizations at weekly intervals, serum was collected and tested in the presence of rabbit complement for cytotoxicity against YAC-1. The serum was diluted 1:6 and absorbed with graded cell numbers at room temperature for 1 hr. Subsequently, 20 μl antiserum dilution were mixed with 5 μl cell suspension containing 10^7 cells/ml. The mixture was incubated for 20 min at 37°C, whereafter the cells were spun down and washed once.

Thirty μl of fetal rabbit serum were added as complement, at a dilution that was non-toxic for untreated control targets. The suspension was incubated for 45 min at 37°C, whereafter cytotoxicity was determined by trypan blue exclusion test. A cytotoxic index was calculated according to the following formula (the control designates cells incubated with complement but without antiserum):

$$\text{C.I.} = \frac{\%\text{ viable cells in control} - \%\text{ viable cells in test}}{\%\text{ viable cells in control}}.$$

Statistical analysis

The frequency of takes in F_1-hybrids was compared with the corresponding frequency in the parental strain by the chi-square test.

RESULTS

Immunoselected sublines

The immunoselected YAC–IR and YAC462–IR sublines have a reduced surface MCSA expression and almost complete resistance to the cytotoxic effect of anti-MCSA serum + complement [16–18]. Prior to the present study, both lines were retested for MCSA expression and compared with the unselected YAC line. Figure 1 shows the results of a quantitative absorption test with graded cell numbers followed by a *C'*-dependent cytotoxicity test against the target YAC-1 *in vitro* line. YAC462–IR showed no significant absorption at the cell concentrations used. YAC–IR showed some absorption, as in previous studies, indicating an approximately ten-fold reduction of its MCSA concentration compared to YAC.

Figure 2 shows the frequency of tumor takes in the syngeneic A/Sn strain and in various semisyngeneic F_1-hybrids after the inoculation of 10^3 YAC, YAC–IR and YAC462–IR cells. In the parental A/Sn strain the take incidence was about the same for all three lines. In agreement with our previous results [4, 6], all tested hybrids except A × ABY were relatively resistant to YAC as compared with A/Sn. Two of the six hybrids resistant to YAC showed a significant reduction of their resistance against YAC–IR. There was an even more pronounced reduction against YAC462–IR. A total abolition of resistance against the latter was only seen in the A × DBA/2 and A × A.CA hybrid combinations.

In an earlier study the two immunoselected YAC–IR and YAC462–IR sublines were as sensitive to NK cells as YAC cells [12].

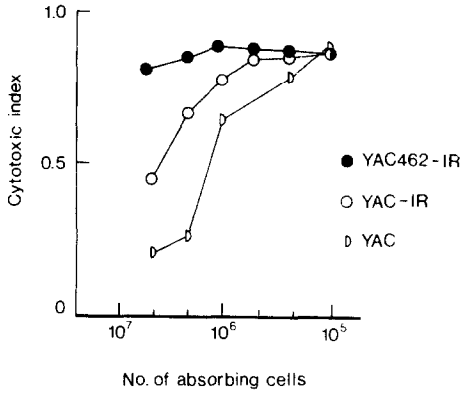

Fig. 1. Quantitative absorption of anti-MCSA serum with YAC, YAC–IR and YAC462–IR. The antiserum diluted 1:6 absorbed with the number of cells ($\log_{10}$ values) indicated in the figure. Complement-dependent cytotoxicity test was performed against YAC-1. Cytotoxic index (C.I.) was calculated according to the formula in Materials and Methods.

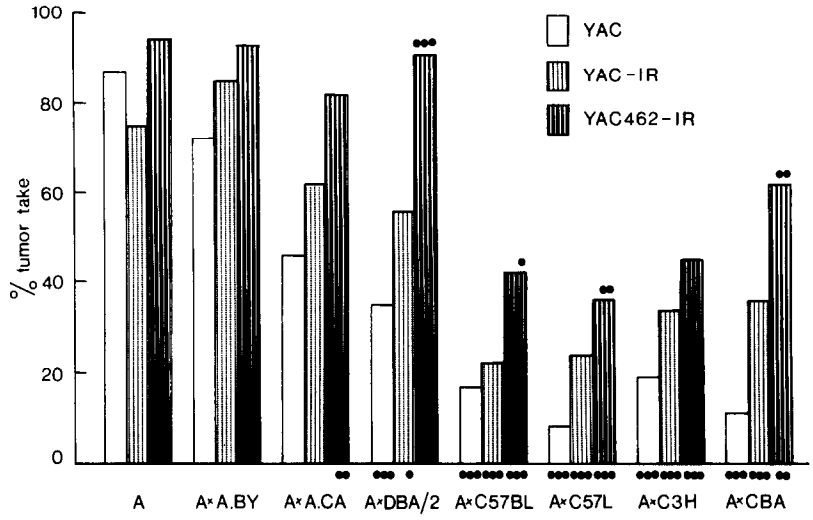

*Fig. 2. Percentage of tumor take in the syngeneic A strain and indicated hybrids after subcutaneous inoculation of 10^3 cells. The statistical significance of the difference between tumor take frequency in the hybrids and in the syngeneic A strain was calculated by χ^2 analysis and is indicated below each bar: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$. The statistical significance of the difference of the take frequency for a particular genotype with the immunoselected sublines YAC-IR and YAC462-IR compared to YAC is indicated above each bar.*

However, we have recently found that this is only true for the *in vitro* cultured lines (Grönberg *et al.*, manuscript in preparation). Recently explanted YAC-IR and YAC462-IR cells showed a decreased NK-sensitivity. Full sensitivity was achieved only after three weeks of tissue culture.

Figure 3 shows a comparison between the sensitivity of the three lines to NK-lysis and to allogeneic cytotoxic *T* cells. C57BL (H-2^b) anti A/Sn (H-2^a) cells were produced by mixed lymphocyte culture and tested in parallel with normal CBA spleen cells. The target lymphoma cells were used directly after their removal from the peritoneal cavity. While the sensitivity of the two immunoselected variants to NK-mediated lysis was considerably reduced, this was not accompanied by any reduction in their sensitivity to the allospecific *T* cells.

In order to compare hybrid resistance and NK-activity, the three lines were also tested for their sensitivity to NK cells from representative F_1-hybrids. As shown in Table 1, YAC-IR and YAC462-IR showed a reduced NK-sensitivity to the killer cells of all tested genotypes.

We have also tested the sensitivity of the three lines to rejection in pre-immunized mice. All mice were immunized three times at weekly intervals with 10^7 6000 rad-irradiated YAC cells intraperitoneally. Viable cell challenge was given one week after the last immunization; all mice received 400 rad whole body irradiation immediately before. Pre-irradiation was introduced because it counteracts the boosting effect of the viable challenge without affecting the pre-existing immune response [23].

As shown in Table 2, A/Sn could not be immunized against any of the three cells. In A × C57L and A × C57BL a strong immunity was induced to YAC, but there was only a slight effect against the two immunoselected sublines. Immunized A × DBA/2 hybrid mice were not protected against YAC or YAC-IR but showed, surprisingly, a certain protection against YAC462-IR. Without immunization, this hybrid showed no resistance to YAC462-IR.

Table 1. NK activity against YAC and immunoselected YAC sublines directly explanted from the peritoneal cavity

Strain	YAC		YAC-IR		YAC462-IR	
	100:1*	33:1	100:1	33:1	100:1	33:1
A/Sn	3.1	2.9	1.1	−0.6	0.7	0.6
A × DBA/2	4.0	2.4	1.5	1.6	−0.3	0.2
A × CBA	6.5	6.9	0.5	−0.9	0.7	1.8
A × C57BL	10.0	7.5	1.9	2.0	1.7	1.9

Percentage lysis was calculated according to formula in Materials and Methods.
*Effector : target cell ratio.

Table 2. Tumor takes in pre-immunized (im) and control (ctrl) mice

cell	dose	A/Sn		A × DBA/2		A × C57L		A × C57BL	
		im	ctrl	im	ctrl	im	ctrl	im	ctrl
YAC	10^3	7/8	8/10	5/8	6/8	0/10	8/10		
YACIR	10^3	6/6	6/6	8/9	7/9	2/6	5/6	1/6	6/6
YAC462IR	10^3	6/6	6/6	2/6	6/6	2/5	5/6	2/6	3/6
YAC	10^4	9/9	9/9	9/12	10/11	0/5	3/5	0/6	6/6
YACIR	10^4	12/12	12/12	10/12	13/13	6/9	9/11	4/12	12/12
YAC462IR	10^4	6/6	6/6	2/6	5/6	4/5	6/6	5/6	6/6

The syngeneic A/Sn and F_1-hybrids were immunized three times with 10^7 cells irradiated with 6000 rad prior to subcutaneous inoculation of viable cells.

Table 3. NK activity against the tissue culture lines YAC-1 and NK-selected variants

Strain	YAC-1		YAC-ACG		YAC-ACK	
	100:1 *	33:1	100:1	33:1	100:1	33:1
A/Sn	10.0	5.5	2.4	2.0	4.1	2.8
A × A.CA	11.0	6.1	1.2	0.2	2.5	1.9
A × DBA/2	17.3	10.1	6.1	4.9	9.4	8.5
A × CBA	25.1	14.9	9.1	5.3	16.0	10.0
A × C57BL	43.4	23.3	16.8	10.5	30.1	16.7

3 Pooled spleens of A/Sn and the indicated F_1-hybrids. Percentage lysis was calculated according to formula in Materials and Methods.
*Effector : target cell ratio.

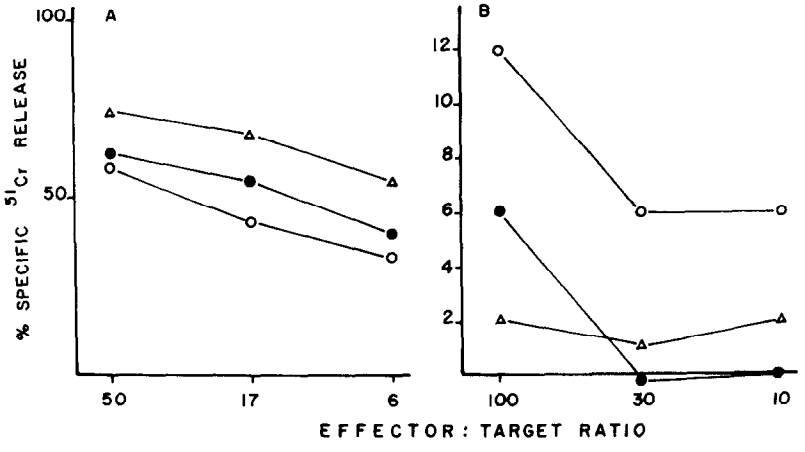

Fig. 3. Sensitivity of the two immunoselected sublines YAC-IR (●—●) and YAC462-IR (△—△), compared to YAC (○—○), for the cytotoxic effect of allospecific T cells (left panel A) and to natural killer cells from CBA. The cytotoxic T cells were C57BL anti-A, generated in vitro by mixed lymphocyte culture as described in Materials and Methods.

The NK-selected sublines

The YAC-ACK8, YAC-ACC10 and YAC-ACG9 lines, selected from the YAC-1 *in vitro* line [19], showed a decreased sensitivity to NK cells compared to YAC-1, although they were not totally insensitive. They showed an unchanged or somewhat increased sensitivity to lysis by allospecific cytotoxic T cells. Quantitative absorption of H-2^a specific alloantisera showed no change in the surface concentration of H-2 antigens [19].

Figure 4 shows the take incidence after the inoculation of 10^3 cells into syngeneic A/Sn and various F_1-hybrid mice. The NK-resistant variants YAC-ACC10 and YAC-ACG9 showed an increased incidence of takes in all genotypes tested, whereas YAC-ACK8 gave the same incidence in A/Sn and A × C57BL as YAC-1. In

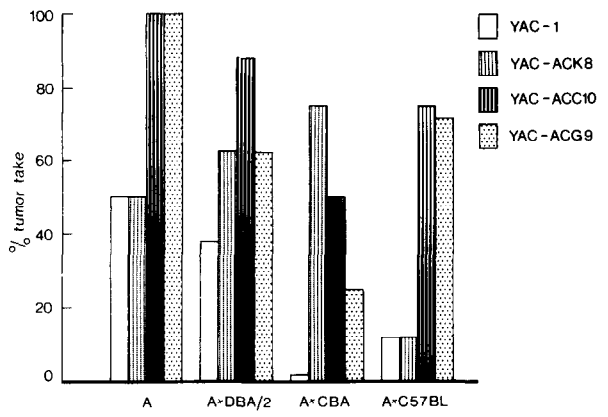

Fig. 4. Percentage tumor take after subcutaneous inoculation of YAC-1 and the three NK-selected sublines. Ascitic cells (10^5) were used for all lines.

spite of the fact that the cell culture lines were passaged twice in the ascitic form *in vivo* before the actual subcutaneous test, YAC-1 frequently regressed after two weeks. The NK-selected sublines also regressed occasionally, but less frequently than YAC-1. The difference between YAC-1 and these sublines was therefore more pronounced if the numbers of mice dying with progressively growing tumors are compared (data not shown).

The decreased sensitivity of the NK-selected variants to NK-lysis was not restricted to the CBA genotype previously tested [19], but was also seen in the AF_1 hybrids tested in the present study. Table 3 shows the results of an experiment with 3 pooled spleens from A, A × CBA, A × DBA/2, A × C57BL and A × A.CA. The decreased sensitivity of the variants to NK-lysis was evident with the effector cells of all genotypes. The YAC-ACG9 line showed the most pronounced difference in this experiment.

DISCUSSION

Previously, we have found a good correlation between the natural killer (NK) activity of various semisyngeneic F_1 hybrids and segregating backcross mice against the YAC lymphoma and their *in vivo* resistance to small tumor inocula, suggesting that NK cells mediate hybrid resistance against this tumor [6, 7]. This conclusion was reaffirmed by the increased incidence of takes of NK-sensitive lymphoma cells in NK-deficient beige mutants, compared to corresponding wild-type hosts [11, 12].

The present study focuses on the question of whether the NK-sensitivity of the YAC lymphoma can be modulated by selection, so as to become increasingly refractory to NK-mediated *in vivo* resistance. Two types of variant sublines were compared. YAC-IR and YAC462-IR have been specifically immunoselected by repeated exposure to anti-MCSA antibody in the presence of complement, alternating with repeated passage in mice pre-immunized with irradiated YAC cells [16, 18]. The resulting variants were more or less completely resistant to the cytotoxic effect of anti-MCSA antibody. Using quantitative absorption experiments, we have confirmed the previously demonstrated decrease of MCSA in one subline and the disappearance of detectable MCSA in the other [17, 18].

We have also shown that both immunoselected lines have a decreased sensitivity to natural killer cells compared to the original YAC line. Their sensitivity to allospecific CTL was unchanged.

The fact that both immunoresistant lines were relatively refractory to the natural resistance of different semisyngeneic F_1 hybrids is in line with the conclusion that this resistance is mediated by NK cells.

The decreased sensitivity of these lines to anti-MCSA antibodies must be considered as well; however, earlier experiments showed that the anti-MCSA antibody formation and NK activity were under independent genetic control [24]. This argues against a protective role of antibodies in this system.

Natural antibodies (NAb) may also be considered as possible mediators of tumor cell rejection [25]. Non-immunized mice of most strains have natural cytotoxic antibodies against YAC cells [26]. We have recently found that the immunoselected YAC-IR and YAC462-IR sublines bind little or no natural antibody by direct cytotoxicity or absorption tests [26]. The specificity of the NAb anti-YAC response is not yet known. It is possible that it represents a natural anti-MCSA response, perhaps due to the horizontal spread of Moloney virus or some related ecotropic murine retroviruses.

The possible participation of NAb in hybrid resistance against YAC is made less likely, however, by the fact that A/Sn mice have similar levels of NAb the more highly resistant A F_1-hybrids [26].

The second group of YAC variants were selected by repeated *in vitro* exposure to NK cells from the highly active (A × CBA) F_1-hybrid, alternating with *in vivo* passage in highly NK-reactive F_1-hybrids. All three variants were relatively, although not completely, insensitive to NK cells.

The NK-selected variants showed no significant decrease of their MCSA expression, in contrast to the immunoselected variants (data not shown). Their decreased sensitivity to

the natural resistance of certain F_1 genotypes *in vivo* is therefore probably a direct effect of their decreased sensitivity to natural killer cells.

It is well-known from other studies that selective pressures against phenotypic tumor cell markers may favor variants that are structurally and/or functionally defective with regard to the corresponding marker.

The present study shows that selection against NK-sensitive target sites may lead to the establishment of relatively NK-resistant sublines that are also less sensitive to hybrid resistance *in vivo*. This reaffirms the previous conclusion that hybrid resistance is mediated by NK cells, at least as far as the YAC lymphoma is concerned.

Selection against the virally determined MCSA has led to variants with decreased or undetectable MCSA expression, as judged by the sensitive absorption method. Interestingly, these variants were also relatively resistant to NK-mediated lysis *in vitro* (if tested directly from the ascites from). They were also resistant to natural cytotoxic antibodies. This finding may indicate a relationship between MCSA and the target antigen recognized by NK cells and NAb on the YAC cell membrane. Previous findings have suggested that the specificities recognized on YAC cells by murine NK cells and by natural antibodies in normal rabbit [27] and in normal mouse serum [28] were identical or related. Our present finding that the immunoselected YAC variants were less sensitive to the hybrid resistance of certain genotypes stresses the need to analyse the cell membrane structures responsible for this difference, and particularly the relationship between MCSA and the target structure for NK cells and natural antibodies.

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