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Induction of Tumor Cell Rejection in the Low Responsive YAC-Lymphoma Strain A Host Combination by Immunization with Somatic Cell Hybrids*

GEORGE KLEIN and EVA KLEIN

Department of Tumor Biology, Karolinska Institutet, S-104 01 Stockholm 60, Sweden

Abstract—*X-irradiated Moloney virus induced YAC lymphoma cells of strain A origin induced only a weak rejection reaction and low antibody levels against living YAC cells in syngeneic strain A mice, in spite of their high MCSA-antigenicity in humoral antibody tests. Fusion with some established mouse fibroblast lines provided somatic cell hybrids that could overcome this limitation: irradiated hybrid cells induced an efficient rejection reaction against YAC in A mice. Other somatic cell hybrids and allogeneic, Moloney virus induced mouse lymphomas were inefficient. Possible relationships between the rejection and humoral antibody inducing abilities of the different hybrid cells were considered.*

INTRODUCTION

INOCULATION of the Moloney-virus (MLV) induced YAC lymphoma of strain A origin [1] induces both antibodies and rejection response against the MLV induced surface antigen (MCSA) in certain semisyngeneic F_1 hybrid mice (e.g., A × C57Bl, A × C57leaden, A × CBA). It is poorly immunogenic in the syngeneic strain, A and some other F_1 hybrids (e.g., A × ASW, A × ACA) [1–3]. The spleen cell population of certain unmanipulated F_1 hybrids is cytotoxic to cultured YAC cells *in vitro* [4], a phenomenon that is designated as the "natural killer" (NK)-cell activity. Spleen cells of other F_1 hybrids and of the syngeneic A host have only weak or no activity in the NK-test. NK-activity was found to be correlated with resistance against the inoculation of small numbers (10^3–10^4) of YAC cells [5]. Backcross tests indicated that both effects (cytotoxicity and resistance) were under genetic control, with a relatively strong H-2 linked resistance factor [6]. The induction of serum antibody was also found to be under the influence of a major dominant gene. This was not identical with the gene(s) regulating NK cell activity, however, and was not linked to H-2 [3].

We have proposed [7] that Ir or Ir-like genes play an important role in the immunity against tumor associated antigens. According to this view, the strong immune surveillance mechanisms that were demonstrated to operate against many of the virus induced tumors, have evolved through the selection of the natural host species for Ir gene mediated rejection responses, directed against antigens associated with potential neoplastic cells induced by the virus. As a rule, the efficiency of the resistance is proportional to the ubiquitousness of the virus. Polyoma in mice, FeLV in cats, *H. saimiri* in the squirrel monkey and EBV in man can be quoted as examples [8].

In contrast, the evidence for immune surveillance against chemically induced or spontaneous tumors is weak or non-existent [7, 9]. This may be attributed to the absence of comparable host selection, rather than the absence of tumor specific or tumor associated membrane changes, potentially capable of eliciting a rejection response on the appropriate genetic background.

If this view is correct, mobilization of rejection responses against tumor associated membrane changes becomes a problem of overcoming genetic unresponsiveness, rather

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than correcting the breakdown of an immune response that may never have existed. The problem is somewhat analogous to the overcoming of genetic unresponsiveness in fundamental immunology, although with some important differences. Immunologists usually depart from a well defined immune response, search for the rare unresponsive individual, explore the genetics and the mechanism of unresponsiveness, and seek to overcome it by administering the unrecognized moiety after chemical coupling to, or together with a well recognized antigen (for reviews see [10, 11]). In the case of the spontaneous or chemically induced tumor we are dealing with the mirror image of this situation. The natural history of most tumors reflects a multistep evolution, designated by the generic name "tumor progression" [12]. The successive selection for increasing independence from growth restricting mechanisms involves, in all probability, selection for non-immunogenic and/or non-rejectable tumor cells. As a consequence, we may have to face a wide variety of neoplasm-associated membrane changes that have been preselected for non-recognition (or no rejection, in spite of recognition) in the given host, and we shall have to deal with the problem of inducing efficient responses in spite of this situation.

Antigenic modification of the tumor cell is one of the possible ways to overcome unresponsiveness and induce rejection. Hapten coupling is one possibility. In a previous study [2] we have found that immunization with TNP coupled YAC cells can induce at least some humoral antibody response and the rejection of relatively small cell numbers in the low-responsive strain A host. Another approach would be the "xenogenization" of the established tumor, by superinfection with another, highly antigenic virus, as performed in other tumor systems [13–15].

A third possibility is to introduce the poorly recognized tumor antigen into a somatic cell hybrid by fusing the target tumor cell with a highly antigenic (allogeneic or xenogeneic) partner. Several authors have shown that both virally and chemically induced, tumor associated antigens can be expressed on somatic cell hybrids of this type [16–22]. Watkins and Chen [23] concluded that hybrid cells are more immunogenic than the parental tumor cell whereas Satya Murthy *et al.* [24] found no increase in immunogenicity. Both groups used interspecies hybrids, but different tumor-partner cell combinations.

The purpose of the present study was to explore the feasibility of overcoming host unresponsiveness in the genetically and antigenically well defined YAC-strain A system. Compared to the previous studies this system appeared to offer the following advantages:

(a) A relatively strong virus induced tumor associated transplantation antigen (MCSA) can be demonstrated on the target YAC cell;

(b) F_1 hybrid hosts of certain genotypes can respond to this tumor, whereas the syngeneic A strain and some other F_1 hybrids do not respond well;—responsiveness can be thus directly related to host genetics;

(c) Successful attempts to overcome the low responsiveness of the syngeneic hosts may have a direct bearing on the question how to mobilize host responses against an existent, but normally poorly recognized tumor associated antigen.

MATERIALS AND METHODS

Cell lines and mice

Table 1 summarizes the origin and characteristics of the cells and hybrids used in the present study. All hybrids were maintained as monolayer cultures on RPMI 1640 medium, with 10% fetal calf serum. The tumor lines were maintained by serial intraperitoneal passage of ascitic fluid in the syngeneic host. For all *in vivo* rejection tests and most of the experiments concerned with antibody formation after immunization, strain A/Sn mice were used. In some antibody tests, (A $\times$ C57leaden)F_1 hybrid mice were included for comparison.

Experimental design

The mice were immunized with 6000 rad irradiated cells at biweekly intervals. Each immunizing inoculation contained between 5×10^6 and 10^7 cells. The cells used for immunization are listed in Table 1. Two weeks after the fifth and last immunization all mice were bled for anti-MCSA antibody titration. One day later they received 400 rad whole-body X-irradiation and were immediately thereafter challenged with live YAC cells, given subcutaneously. Whole body irradiation was considered necessary like in previous studies [25], to distinguish between an established immunity, resistent to 400 rad and a non-specific boostering of the primary immune response (sensitive to 400 rad). Tumor growth was followed by caliper measurements, twice weekly.

In certain experiments, immunized mice

Table 1. *List of cell lines**

Moloney virus induced lymphoma line	Genotype	Hybridized with	Known antigens introduced by partner cell	Reference
YAC	H-2^a	A9†	H-2^k, L virion, L-cell	(26)
YAC	H-2^a	A9HT‡	L virion	(30)
YACIR§	H-2^a	A9†	L virion¶	(26)
YACIR	H-2^a	A9HT‡	L virion¶	(30)
YACIR	H-2^a	MSWBS‖	H-2^s, MC-TSTA	(31)
YACIR	H-2^a	Normal CBA T 6T 6 fibroblast	H-2^k	(31)
YBA	H-2^k (CBA)	—	—	—
YBB	H-2^k (CBA) H-2^a H-2^b	—	—	—
YALB	(A × C57leaden) H-2^a H-2^b	—	—	—
YA7C	(A × C57B1)	—	—	—

*The production and properties of the cell hybrids used in this study have been described in references [30–32].
†Low malignant L-cell subline.
‡High malignant L-cell subline.
§Immunoresistant subline of YAC, with reduced MCSA concentration [32].
¶In the YAC/A9 and YACIR/A9 hybrids, the characteristic high MCSA concentration of YAC was reestablished [18].
‖Ascitic form of methylcholanthrene induced sarcoma [21].

were followed by regular weekly bleedings for cytotoxic antibodies, in parallel with tumor size measurements.

Cytotoxicity tests

A complement mediated microcytotoxicity assay was performed in the following way:

Thirty ring microplates (Möller and Coates, Moss, Norway) were filled with 5.5 ml liquid paraffin. Sera were diluted in 2-fold steps with balanced salt solution (BSS) containing 1% gelatin. A droplet of 2 μl serum dilution was placed on the glass bottom of the microplate under paraffin oil with the aid of a Hamilton syringe. A control anti-Moloney serum and a normal control serum were always included in each plate. The plates were then kept at −20°C until use. YAC ascites cells were harvested 7–8 days after intraperitoneal inoculation of 10^6 live YAC cells. The ascites fluid was centrifuged and cells washed 3 times with RPMI medium containing 10% fetal calf serum. For the cytotoxic test, plates were thawed by 2–3 min incubation at 37°C and 10^3 YAC ascites cells in a total volume of 1 μl were added to the serum droplet and incubated for 20 min at 37°C. Thereafter 1 μl of rabbit complement diluted 1:10 with RPMI 10% FCS was added and the plate was gently shaken to allow complement to spread uniformly. After further incubation for 40 min at 37°C 0.5 μl trypan blue was added (0.025 g trypan blue in 5 ml 0.9% NaCl at pH 7 freshly prepared and kept at 37°C). Plates were then read under the microscope and 100 cells counted. Background non-specific killing of normal serum usually ranged between 2 and 10%.

RESULTS

Groups of 3 strain A mice were immunized with irradiated cells of the syngeneic Moloney lymphoma YAC, or the allogeneic (CBA-derived) Moloney lymphoma YBA, or similarly irradiated somatic cell hybrids of the types YACIR/A9 and YACIR/A9HT, respectively. Two weeks after the last immunization, all mice were irradiated with 400 rad and challenged with 10^2, 10^3 and 10^4 viable YAC cells, respectively, in parallel with untreated controls, Both YACIR/A9 and YACIR/A9HT induced protection whereas immunization with YAC cells had only minimal effect and YBA cells had no detectable effect.

From groups of 10 A mice immunized with irradiated cells, equal amounts of sera were pooled for each group and tested for cytotoxicity against YAC target cells. Neither YAC nor YBA induced detectable antibodies (titer <2) whereas the somatic hybrids and the (A $\times$ C57Bl)F_1 lymphoma YA7C induced significant antibody titers.

With some of the cells (A $\times$ C57leaden)F_1 mice were also immunized. In this case, antibodies appeared earlier and reached the higher titers than in A mice. Moreover, the radiated YAC cells were also capable to induce antibody formation in this F_1 hybrid.

The immunity against subcutaneously grafted YAC cells varied according to the cell used for immunization. Figure 1 summarizes all experiments that were carried out with the same design, in 2 or 3 parallel series for each immunizing hybrid-target cell combination. YAC had a slight immunizing effect only. Immunization with YACIR/A9HT was most efficient, detectable both by an increased incidence of rejection with all three cell doses, increased survival time of the tumor bearing animals, and the highest antibody levels. The latter was found both in the syngeneic A mice and in the (A $\times$ C57leaden)F_1 hybrid (compare legend of Fig. 1).

Lower degrees of immunization were achieved with the other somatic cell hybrids. They appeared to be immunogenic, to some extent at least, as reflected both by rejection and by antibody titers, but appeared to be intermediate between the low immunogenic YAC and the relatively highly immunogenic YACIR/A9HT.

In addition to the somatic hybrids, allogeneic Moloney virus lymphomas were also tested (not shown on the curves). Immunization with irradiated cells of the Moloney virus induced YBB lymphoma of CBA origin, YALB lymphoma of (A $\times$ C57leaden)F_1 origin or YA7C lymphoma of (A $\times$ C57Bl)F_1 origin had no discernible protective effect against 10^2, 10^3 or 10^4 YAC cells in strain A mice.

DISCUSSION

Somatic cell hybrids, derived from the fusion of YAC with an unrelated normal or malignant mouse cell that introduced new, genetically or virally determined antigens, were immunogenic in strain A mice, as reflected both by the rejection and the cytotoxicity tests. In this strain, YAC cells are either non-immunogenic or induce only very low levels of antibodies and rejection responses. All somatic cell hybrids were efficient, although to a variable degree. YACIR/A9HT

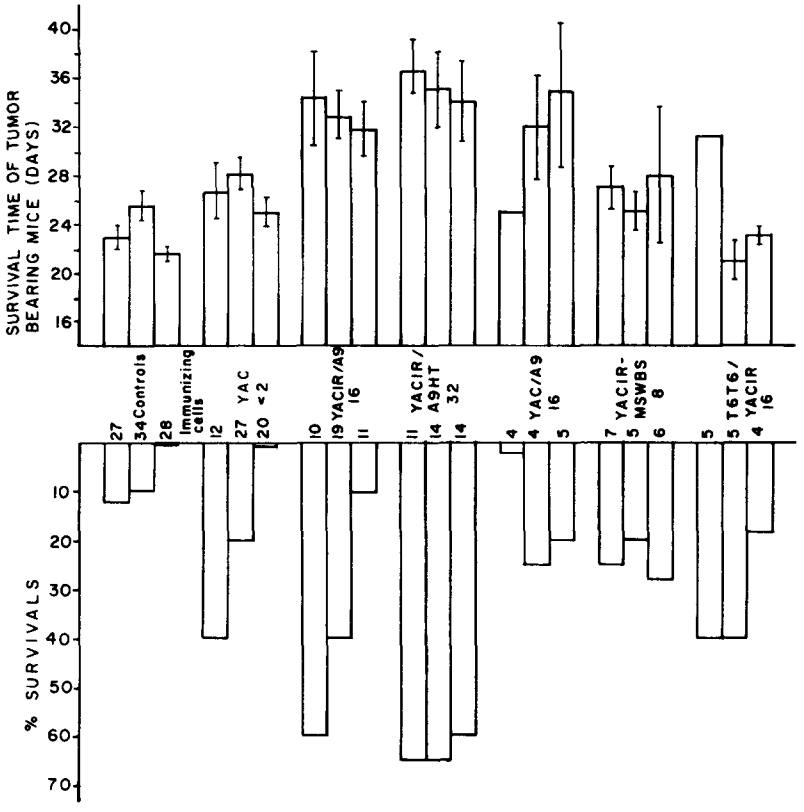

Fig. 1. *Frequency of survival and mean survival times of tumor bearing mice, after immunization with heavily irradiated YAC cells or various somatic cell hybrids as indicated. For each group the three columns show the results after the inoculation of* 10^2, 10^3 *and* 10^4 *viable YAC cells to strain A mice that have been preimmunized with the cells indicated. All hosts were irradiated with* 400 rad *prior to the viable inoculum. The figures in the middle, under each immunizing cell designate the mean antibody titers in strain A mice, as measured by cytotoxic tests. After immunization with the identical schedule, the mean antibody titers induced in* $(A \times C57leaden)F_1$ *mice were* 32 *for YAC,* 64 *for YACIR/A9 and* 256 *for the YACIR/A9HT immunization group. The figures at the foot of the columns indicate the number of mice used for each experiment.*

was the most immunogenic hybrid, both by humoral antibody formation and graft resistance. The other somatic hybrids were intermediate. Allogeneic Moloney lymphomas did not induce detectable rejection responses, although they did induce variable antibody levels.

The mechanism that determines the variation in the ability of the different hybrids to induce rejection reactions is unknown. Since hybrids derived from basically similar partner cell combinations (e.g., YAC/A9, as compared to YACIR/A9 and YACIR/A9HT) showed differences in this respect, it must be assumed that their antigen expression or presentation was different. Somatic cell hybrids were indeed found to vary in their antigen expression [18, 26, 27]. Further studies will be performed to establish whether the relevant parameter can be pinpointed as one of the serologically detectable (e.g. MCSA) antigens or other phenotypic or karyotypic properties.

Recent studies have shown that the YAC lymphoma is uniquely susceptible to the natural killer (NK) cell that can mediate resistance against "small" inocula in non-preimmunized mice of certain genetic constitutions [4–6, 7]. In contrast, YAC cells show little or no susceptibility to semi-syngeneic $(A \times C57B1)F_1$ T-cells in MLV or MSV immunized syngeneic mice, although these T-cells were highly cytotoxic for the Rauscher-virus induced lymphoma line (RBL) [28]. This suggests a difference in the susceptibility of different cell lines induced by the same or antigenically closely related viruses to different immune effectors. The ability of YACIR/A9HT and other somatic hybrids to induce an efficient anti-YAC rejection in syngeneic A mice deserves further study with regard to the effector mechanisms involved.

Viewed in a more general context, antigenic modification of tumor cells by somatic cell hybridization appears to be one of the poten-

tially rewarding pathways that can render a relatively non-immunogenic tumor highly immunogenic. The existence of "universal fuser" -cells, e.g., cell lines unable to grow on HAT but sensitive to ouabain [29] makes this approach eminently practical, since a single cell line that carries the appropriate markers can be readily fused with any murine or human tumor cell. The selection of universal fusers that are outstanding in rejection-induced experiments of the type described in this paper appears as an importent task for the future.

REFERENCES

1. G. Klein, E. Klein and G. Haughton, Variation of antigenic characteristics between different mouse lymphomas induced by the Moloney virus. *J. nat. Cancer Inst.* **36,** 607 (1966).
2. N. Galili, D. Naor B. Åsjö, and G. Klein, Induction of immune responsiveness in a genetically low-responsive tumor-host combination by chemical modification of the immunogen. *Europ. J. Immunol.* **6,** 473 (1976).
3. B. Åsjö, R. Kiessling, G. Klein and S. Povey, Genetic variation in antibody response and natural killer cell activity against a Moloney virus-induced lymphoma (YAC). *Europ. J. Immunol.* **8,** 554 (1977).
4. R. Kiessling, E. Klein and H. Wigzell, "Natural" killer cells in the mouse. I. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Specificity and distribution according to genotype. *Europ. J. Immunol.* **5,** 112 (1975).
5. R. Kiessling, G. Petranyi, G. Klein and H. Wigzell, Genetic variation of *in vitro* cytolytic activity and *in vivo* rejection potential of non-immunized semisyngeneic mice against a mouse lymphoma line. *Int. J. Cancer* **15,** 933 (1975).
6. G. Petranyi, R. Kiessling, S. Povey, G. Klein, L. Herzenberg and H. Wigzell, The genetic control of natural killer cell activity and its association with *in vivo* resistance against a Moloney lymphoma isograft. *Immunogenetics* **3,** 15 (1976).
7. G. Klein, Immunological surveillance against neoplasia. *Harvey Lect.* **69,** 71 (1975).
8. G. Klein and E. Klein, Immune surveillance against virus-induced tumours and nonrejectability of spontaneous tumours: contrasting consequences of host versus tumor evolution. *Proc. nat. Acad. Sci. (Wash.)* **74,** 2121 (1977).
9. H. B. Hewitt, E. R. Blake and A. S. Walder, A critique of the evidence for active host defence against cancer, based on personal studies of 27 murine tumours of spontaneous origin. *Brit. J. Cancer.* **33,** 241 (1976).
10. B. Benacerraf and H. O. McDevitt, Histocompatibility-linked immune response genes. *Science* **175,** 273 (1972).
11. G. M. Shearer, E. Mozes and M. Sela, Cellular basis of the genetic control of immune responses. In *Progress in Immunology.* (Edited by D. B. Amos) p. 509. Academic Press, New York (1971).
12. L. Foulds, The natural history of cancer. *J. chron. Dis.* **8,** 2 (1958).
13. H. Kobayashi, F. Sendo, T. Shirai, H. Kaji, T. Kodama and H. Saito, Modification in growth of transplantable rat tumors exposed to Friend virus. *J. nat. Cancer Inst.* **42,** 413 (1969).
14. J. Lindenmann and P. A. Klein, Viral oncolysis: increased immunogenicity of host cell antigen associated with influenza virus. *J. exp. Med.* **126,** 93 (1967).
15. G. J. Svet-Moldavsky and V. P. Hamburg, An approach to the immunological treatment of tumors by artificial heterogenization. In *Specific Tumor Antigens.* UICC Monograph Series 2. p. 323. Munksgaard, Copenhagen (1967).
16. G. Barski, M. G. Blanchard, J. K. Youn and B. Leon, Expression of malignancy in interspecies Chinese hamster X mouse Cell. *J. nat. Cancer inst.* **51,** 781 (1973).
17. V. Defendi, B. Ephrussi and H. Koprowski, Expression of polyoma-induced cellular antigen(s) in hybrid cells. *Nature (Lond.)* **203,** 495 (1964).
18. E. M. Fenyö, G. Grundner, E. Klein and H. Harris, Surface antigena and release of virus in hybrid cells produced by the fusion of A 9 fibroblasts with Moloney lymphoma cells. *Exp. Cell Res.* **68,** 323 (1971).
19. J. Jami and E. Rietz, Expression of tumor-specific antigens in mouse somatic cell hybrids. *Cancer Res.* **33,** 2524 (1973).

20. G. KLEIN and H. HARRIS, Expression of polyoma induced transplantation antigen in hybrid cell lines. *Nature New Biol.* **237,** 163 (1972).
21. G. KLEIN and E. KLEIN, Are methylcholanthrene-induced sarcoma-associated, rejection-inducing (TSTA) antigens, modified forms of H-2 linked determinants? *Int. J. Cancer* **15,** 879 (1975).
22. W. LIANG and E. P. COHEN, Resistance to murine leukemia in mice rejecting syngeneic somatic hybrid cells. *J. Immunol.* **116,** 623 (1976).
23. J. F. WATKINS and L. CHEN, Immunization of mice against Ehrlich ascites tumors using a hamster/Ehrlich ascites tumor hybrid cell line. *Nature (Lond.)* **223,** 1018 (1969).
24. M. SATYA-MURTHY, J. BELCHRADEK and G. BARSKI, Interspecies mouse X akodon urichi somatic cell hybrids: comparative immunogenicity of parental and hybrid cells. *Europ. J. Cancer* **12,** 33 (1976).
25. G. KLEIN and E. KLEIN, Antigenic properties of other experimental tumors. *Cold Spr. Harb. Symp. quant. Biol.* **27,** 463 (1963).
26. G. KLEIN, U. BREGULA, F. WIENER and H. HARRIS, The analysis of malignancy by cell fusion. I. Hybrids between tumor cells and L cell derivatives. *J. Cell Sci.* **8,** 659 (1971).
27. G. KLEIN, S. FRIBERG, JR. and H. HARRIS, Two kinds of antigen suppression in tumor cells revealed by cell fusion. *J. exp. Med.* **135,** 839 (1972).
28. S. BECKER and E. KLEIN, Decreased "natural killer" effect in tumor-bearing mice and its relation to the immunity against oncorna virus-determined cell surface antigens. *Europ. J. Immunol.* **6,** 892 (1976).
29. H. L. OZER and K. K. JHA, Malignancy and transformation: expression in somatic cell hybrids and variants. *Advanc. Cancer Res.* **25,** 53 (1977).
30. F. WIENER, G. KLEIN and H. HARRIS, The analysis of malignancy by cell fusion. IV. Hybrids between tumor cells and malignant L cell derivatives. *J. Cell Sci.* **12,** 253 (1973).
31. F. WIENER, G. KLEIN and H. HARRIS, The analysis of malignancy by cell fusion. IV. Hybrids between different tumour cells. *J. Cell Sci.* **16,** 189 (1974).
32. E. M. FENYÖ and G. KLEIN, Independence of Moloney virus-induced cell-surface antigen and membrane-associated virion antigens in immunoselected lymphoma sublines. *Nature (Lond.)* **260,** 355 (1976).