TWO KINDS OF ANTIGEN SUPPRESSION IN TUMOR CELLS REVEALED BY CELL FUSION*

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In a previous study, the expression of certain murine cell surface antigens was measured in hybrid cells derived from the fusion of various normal and malignant cells (1, 2). As a rule, the antigens contributed by both parental cells were expressed in the hybrid cell in a codominant fashion, as in cells from heterozygous animals produced by normal genetic crossing. There was one notable exception to this rule. All hybrids produced by the fusion of an Ehrlich ascites tumor cell with other partners showed some suppression of the antigens introduced into the hybrid cell by the other partner. This was true both for the genetically defined antigens of the *H-2* complex and for non-*H-2* antigens such as the surface antigen (FMR type), determined by a C-type virus carried by the L cell line, and the L antigen, another antigenic marker, specific for the L cell. Progressive chromosome loss occurs during prolonged passage of these hybrid cells in vitro or in vivo, and associated with this loss, the initial antigenic suppression may be abolished. In several sublines produced in this way the missing antigenic phenotype was reacquired fully or in part (2, 3).

The suppression of surface antigens in the Ehrlich hybrids was thus "dominant." The fact that antigens determined in very different ways were suppressed en bloc suggested that the suppression mechanism might operate at a rather peripheral level, perhaps by the deposition of some masking substance on the outer cell membrane. The Ehrlich tumor has been selected for progressive growth by passage in genetically foreign (allogeneic) mice for 75 yr. The selection pressure favoring cells with reduced antigen expression was therefore extreme in this cell line. The question arises whether immunoresistance in other cell lines, not selected in this way, is produced by mechanisms similar to those operating in the Ehrlich tumor.

To examine this question, we tested antigenic expression in another series of hybrid cells. The mammary carcinoma TA3, of strain A origin, was chosen for study because there are two sublines of it that differ greatly in the concentration of $H\text{-}2^a$ antigen they contain and in their homotransplantability. The tumor arose in a strain A/HeHa mouse in Dr. T. S. Hauschka's laboratory in 1949 (4) and was subsequently converted to the ascites form (5). One ascites subline was brought to Stockholm while the other was passaged further by Dr. Hauschka. The Stockholm line (designated TA3/St) has maintained a high $H\text{-}2^a$ expression and is readily rejected by allogeneic mice. The

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American line (designated TA3/Ha) has a reduced *H-2*^{a} concentration and grows in genetically foreign strains of mice. Details of the cytological and biological characteristics of the two TA3 lines will be published elsewhere.[1, 2] Unlike the Ehrlich tumor, the TA3/Ha line has never been subjected to any known selection for growth in allogeneic hosts. Its antigen expression and transplantation specificity decreased, for unknown reasons, during serial passage of the tumor in syngeneic strain A mice (T. S. Hauschka, personal communication).

In the course of a study concerned with the expression of malignant behavior in hybrid cells derived from the fusion of normal and malignant cells (6), a series of hybrid clones was produced by fusing the TA3/Ha cell with diploid fibroblasts explanted directly from embryonic ACA (*H-2*^{f}) mice. Quantitative studies on the *H-2* antigen expression of the hybrids are described in the present paper.

Materials and Methods

Cells.—The origin of the TA3 mammary tumor, derived from a strain A/HeHa breeding female in Dr. T. S. Hauschka's laboratory in 1949, has been described (4). The tumor was converted to the ascites form on two separate occasions and both lines were carried by serial intraperitoneal passage in syngeneic recipients. The subline designated TA3/St was brought to Stockholm by one of us (G. K.) in 1951, whereas the other line, designated TA3/Ha, was maintained by Dr. T. S. Hauschka at the Roswell Park Memorial Institute in Buffalo, N.Y. The Stockholm subline grows progressively in 100% of syngeneic strain A mice and is regularly rejected by allogeneic recipients, even if high challenge doses are inoculated (10^5 and 10^6 cells).[1, 2] The Hauschka subline grows indiscriminately in allogeneic *H-2*–incompatible mice (7); it has lost strain specificity without any known selection pressure. It was received in our laboratory from Dr. T. S. Hauschka in 1969 and was subsequently maintained by serial passage in syngeneic recipients.

The TA3/Ha line was fused with trypsinized mouse embryo fibroblasts of the ACA (*H-2*^{f}) strain as described previously (6). TA3 cells grow well as a monolayer on the plastic surface and they outgrow the hybrid cells in the initial fused cell population. Since no selective procedures were available to isolate the hybrid cells from the parental cells, a large number of clones were grown from the fused cell population. Clones showing a clearly different morphology from the tumor cells or the normal fibroblasts were isolated and grown up as separate subpopulations. A number of them turned out to be hybrid cells on karyological examination. Hybrid cell lines were thus established from the original clones. Clones designated TA3/Ha/ACA clones 2, 3B, 4, 5, 6, 7, 10, 11, and 12 were used for antigenic typing and the results are described in the present paper. Studies on the tumorigenicity of these lines are reported elsewhere.

All hybrid lines were grown as monolayers in plastic flasks on Dulbecco's medium (8) with 10% fetal calf serum and the following antibiotics: penicillin 200 μg, streptomycin 200 μg, kanamycin 30 μg, neomycin 30 μg, and Mycostatin 40 μg/ml (E. R. Squibb and Sons, New York).

Embryonic fibroblasts, derived from strain A, ACA, and (A × ACA)F_1 mice, were used as controls in some of the absorption experiments. For this purpose, 8–14-day-old embryos of

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[1] Friberg, S., Jr. 1972. Comparison of an immunoresistant and an immunosusceptible ascites subline from the murine tumor TA3. I. *J. Nat. Cancer Inst.* In press.

[2] Friberg, S., Jr. 1972. Comparison of an immunoresistant and an immunosusceptible ascites subline from the murine tumor TA3. II. *J. Nat. Cancer Inst.* In press.

the required genotype were decapitated, eviscerated, and cut into small fragments. The pieces were trypsinized (0.25%) for 60 min at 37°C, washed twice, and seeded into 75-cm^2 plastic tissue culture bottles (No. 3024, Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) in Eagle's minimal essential medium supplemented with 10% fetal calf serum. The cells that settled within 24 hr were rinsed with fresh medium and subcultured when confluent. Only second-passage fibroblasts were used in the absorption studies. TA3/St and TA3/Ha cells were established as in vitro lines by explantation of the ascites tumors. The tumor cell lines were maintained in the same way as the fibroblasts.

Antisera.—Isoantisera were produced by immunizing groups of 10–13 adult mice with pools of normal tissues. Cell suspensions prepared from the spleen, kidney, and liver were injected subcutaneously once every 2 wk for 14–24 wk. The mice were bled 7–10 days after the last injection.

To identify K-end antigens of the *H-2*^{a} complex, an ACA × C57BL anti-C3H serum was used. D-end antigens of the *H-2*^{a} complex were identified with a monospecific anti-*H-2*^{d} serum (pool C4) obtained from the Transplantation Reference Center, National Institutes of Health, Bethesda, Md. C4 is a (C3H.SW × AKR.M)F_1 anti-B10.A ([*H-2*^{b} × *H-2*^{m}]F_1 anti-*H-2*^{a}) serum. To identify the ACA-derived *H-2*^{f} antigens, an A anti-ACA serum was used. All sera were stored frozen at −20°C.

Absorption Tests.—To measure the concentration of *H-2*^{a}–determined isoantigens on the surface of the two TA3 sublines, the various TA3/Ha/ACA hybrid clones, and the normal embryonic fibroblasts, quantitative absorption tests were run against standard antiserum–target cell systems.

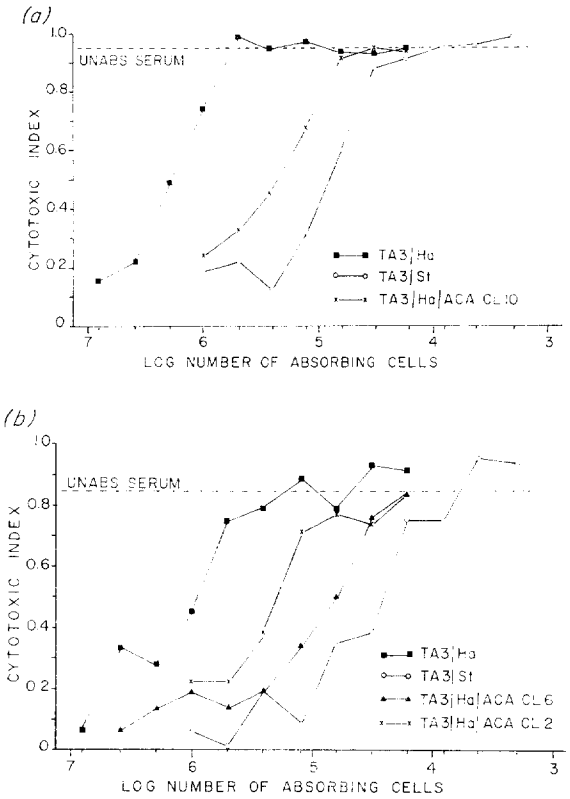
To measure the antigens specified by the K-end of the *H-2*^{a} complex (9), strain A lymphocytes were exposed to an anti-*H-2*^{k} (ACA × C57BL anti-C3H) serum, in the presence of complement, unabsorbed and after absorption with various numbers of test cells. D-end antigens of the *H-2*^{a} complex were measured by testing the C4 antiserum pool against strain A lymphocytes, before and after adsorption. Antigens specified by the ACA-derived (*H-2*^{f}) antigen complex were measured by absorbing the A anti-ACA serum and testing its cytotoxic activity against ACA lymphocytes.

For the absorption test, the two TA3 tumor lines maintained in vitro were harvested by shaking the bottles and centrifuging the suspension. The hybrid cells were harvested with a rubber policeman and used directly. Embryonic fibroblasts were brought into suspension with 0.02% ethylenediaminetetraacetate and filtered through a double layer of gauze to remove cell clumps. All cell suspensions were diluted to yield cell numbers ranging from 10^3 to 10^7. The requisite cell numbers were introduced into small centrifuge tubes. After centrifugation, the supernatants were removed from the tightly packed cell pellets; 25 μl of antiserum at an appropriate dilution was then added to each tube. The unadsorbed antiserum was standardized against the target lymphocytes; the highest dilution that gave a cytotoxic index between 0.8 and 1.0 was chosen as the standard. The working dilution varied between 1:12 and 1:128 with the different antisera. The mixture of cells and antiserum was incubated at 37°C for 45 min. The cells were then removed by centrifugation, and the supernatant was stored frozen at −20°C until required for assay.

Lymph nodes of adult mice of the appropriate strain were minced in a balanced salt solution. Tissue fragments and large clumps of cells were removed by sedimentation. The supernatant, which contained mostly single cells, was transferred to fresh tubes. The cells were washed twice by centrifugation, counted in a hemacytometer, and diluted to give a concentration of 5×10^5 cells/ml. 0.1 ml samples of this suspension were transferred to small centrifuge tubes and spun down. The supernatants were removed and 20 μl volumes of the absorbed or unabsorbed test sera were added to each tube. The cells in the pellets were shaken into suspension and incubated at 37°C for 20 min. They were washed once by centrifugation and deposited to the bottom of the centrifuge tubes. A 30 μl volume of a 1 in 2 dilution of guinea pig complement, previously absorbed with mouse ascites tumor cells, was then added to each

tube. The cells were shaken into suspension and incubated at 37°C for 45 min. 1 drop of trypan blue was then added to each tube and approximately 100 cells were scanned to determine the percentage which had taken up the stain. A cytotoxic index was calculated for each tube by subtracting the percentage of viable cells from the corresponding figure obtained with the control serum and dividing the difference by the latter figure.

In order to compare the absorptive capacity of the various cells for the single pool of



FIGS. 1 *a* and *b*. Absorption of an anti-$H\text{-}2^k$ serum (ACA × C57BL anti-C3H serum, 1:128 dilution) by TA3/Ha, TA3/St, and TA3/Ha/ACA clone 10 (Fig. 1 *a*) and by TA3/Ha/ACA clones 2 and 6 (Fig. 1 *b*). C3H lymphocytes served as target cells for assay of the cytotoxic activity of the absorbed sera. The results are expressed in terms of the cytotoxic index. The activity of the unabsorbed serum is indicated by the dotted line.

antiserum that was used throughout, the ratio of bound to free antibody $(100 - P)/P$ was calculated by the method of Reif and plotted against the ratio (g) of the number of cells to the volume of antiserum used in the absorption (10). A regression line through a double log plot was calculated by the method of least squares, and the value of G ($= g$ when $P = 50\%$) was interpolated. The reciprocal, $1/G$, was used as a single numerical estimate for the absorptive capacity of the different cell types. Values of $100 - P/P$ below 0.1 or above 0.9 were not used in the calculations, because at these levels the error is too high. The values of $1/G$ were calculated for 1 ml of the antiserum at the dilution used.

RESULTS

Fig. 1 illustrates individual absorption experiments done to measure the *H-2*^{a}–derived K-end antigens. Figs. 1 *a* and 1 *b* show two different protocols where the absorptive capacity of the two TA3 lines is compared with hybrid clones 10 (Fig. 1 *a*) and 2 and 6 (Fig. 1 *b*), respectively. There is a more than tenfold difference in the absorptive capacity of the two TA3 lines in both experiments; TA3/St has much more antigen than TA3/Ha. All three clones

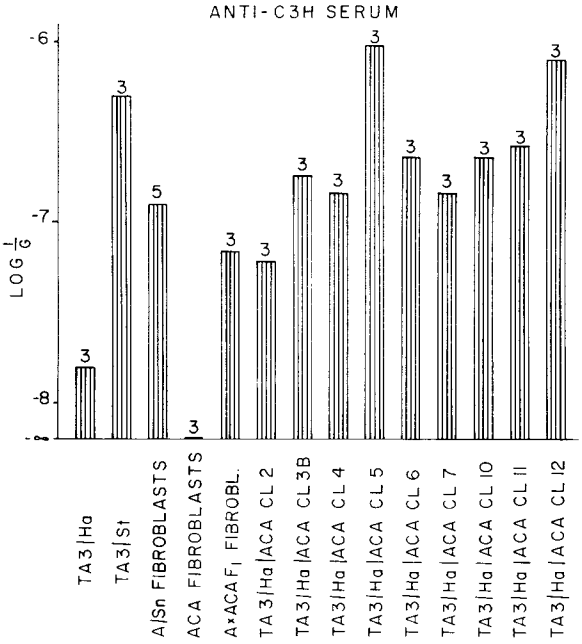


FIG. 2. Absorptive capacity of the TA3/Ha and TA3/St lines, nine different TA3/Ha/ACA hybrid clones, and normal A, ACA, and (A × ACA)F_1 fibroblasts, tested against anti-*H-2*^{k} serum. The $1/G$ values (see reference 10) are plotted on a logarithmic scale and indicate the amount of antibody bound per cell when 50% of the antibody in the serum had been absorbed.

occupy an intermediate position; they do not have the absorptive capacity of TA3/St, but they clearly exceed TA3/Ha. Since fusion with the ACA fibroblast introduces *H-2*^{f} antigens, which cannot cross-react in this absorption system, it must be concluded that the hybrid cells show an increased expression of the K-end antigens of the *H-2*^{a} complex introduced into the hybrid cell by the TA3/Ha parent, despite the fact that the hybrid cell is larger and *H-2*^{a} receptor sites are intermingled with *H-2*^{f} sites.

A more quantitative way of comparing the antigen expression of different cells is provided by the formula of Reif (10). It was used to calculate the ab-

sorptive capacity, $1/G$, which expresses the amount of antibody bound per cell when 50% of the antibody is absorbed. Fig. 2 shows a logarithmic plot of the $1/G$ value for the parental and hybrid lines tested with the anti-$H\text{-}2^k$ serum. Normal parental strain and F_1 fibroblasts are shown for comparison. Each column is based on the results of at least three individual tests, as indicated. TA3/Ha absorbed significantly less than TA3/St. Expressed in terms of cell numbers the difference was 32-fold, i.e., 32 times more TA3/Ha cells were required, on average, to bind the same amount of antibody as a given number

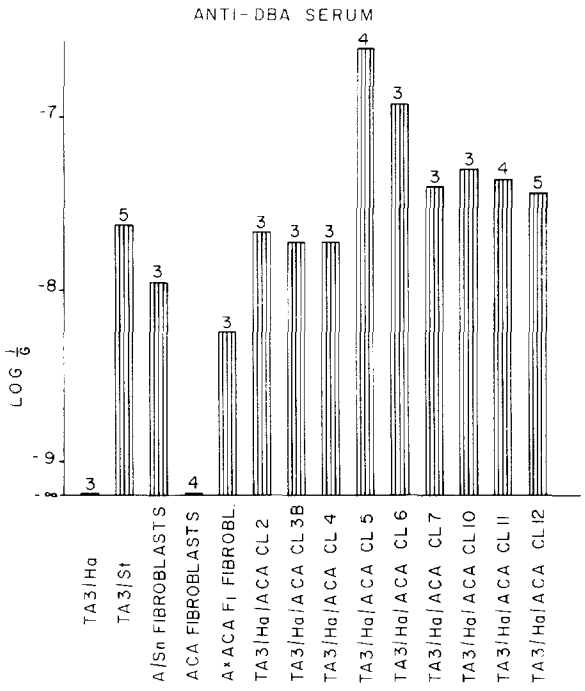


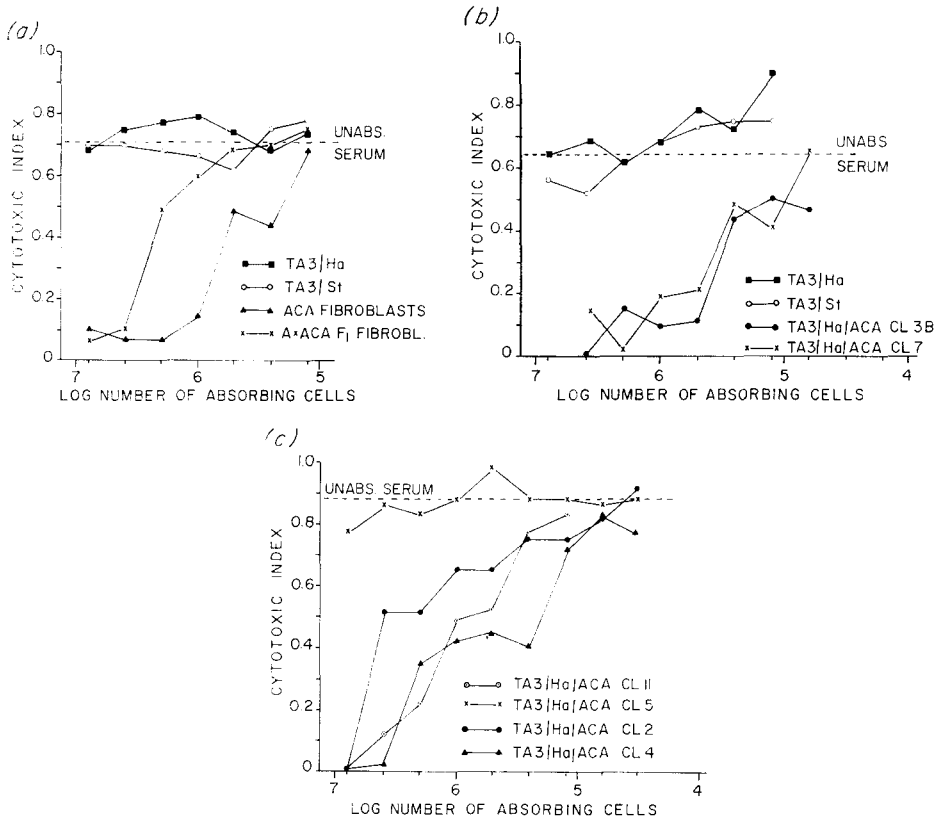
FIG. 3. Absorptive capacity of the TA3 lines, the TA3/Ha/ACA hybrid clones, and the parental and hybrid fibroblasts against anti-$H\text{-}2^d$ serum.

of TA3/St cells. ACA fibroblasts absorbed no significant amount of antibody, as expected. Strain A fibroblasts absorbed 7.5 times more than TA3/Ha and 4.2 times less than TA3/St; (A × ACA)F_1 fibroblasts absorbed 1.8 times less than A fibroblasts. The absorptive capacity of the homozygous TA3/St line was thus approximately seven times higher than that of the F_1 fibroblasts. These results are presumably a reflection of the difference between the number of antigenic receptors on homozygous and heterozygous cells and also of differences in the number of antigenic receptors on different cell types.

Among the TA3/Ha/ACA hybrid lines, the absorptive capacity of clone 2 closely resembled that of the heterozygous fibroblast, whereas all other clones

tested had a higher absorptive capacity. Clones 3B, 4, 6, 7, 10, and 11 were fairly similar; their absorptive capacity was only 2.0–3.3 times less than TA3/St itself. Clones 5 and 12 were exceptional in that their absorptive capacity was of the same order as TA3/St, or even higher.

Fig. 3 summarizes the *H-2*^{d} antigen expression (determined by the *H-2*^{a}



FIGS. 4 *a–c*. Absorption of an anti-*H-2*^{f} serum (A anti-ACA serum, dilution 1:12) by TA3/St, TA3/Ha, ACA, and (A × ACA)F_1 fibroblasts (Fig. 4 *a*) and by six different TA3/Ha/ACA hybrid clones (Figs. 4 *b* and *c*). Normal ACA lymphocytes served as target cells.

complex) of the different cells. ACA fibroblasts carried no *H-2*^{d} antigens as expected. Strain A fibroblasts had 1.9 times more antigen than (A × ACA)F_1 fibroblasts and 2.2 times less than TA3/St cells. TA3/Ha cells did not absorb enough antibody under the test conditions used to allow the calculation of $1/G$; they did, however, carry some *H-2*^{d} antigen since cell doses exceeding 10^7 per sample showed specific removal of anti-*H-2*^{d} antibodies.

All TA3/Ha/ACA hybrid clones tested contained relatively high amounts of *H-2*^{d} antigen, exceeding the levels found in (A × ACA)F_1 fibroblasts or even

homozygous A fibroblasts. Clones 2, 3B, and 4 approached, but did not quite reach, the level as found in the TA3/St line, whereas clones 6, 7, 10, 11, and 12 exceeded these levels. Clone 5 was outstanding; its *H-2*^{d} antigen concentration was 10 times higher than that of TA3/St.

Studies on the expression of the antigens determined by the *H-2*^{f} complex, contributed to the hybrid by the normal ACA fibroblast, are illustrated by Figs. 4 and 5. As expected, the absorption protocol shown in Fig. 4 *a* demonstrates that neither the TA3/St nor the TA3/Ha line can absorb any activity from the anti-ACA serum. ACA fibroblasts absorbed more antibody than (A × ACA)F_1 fibroblasts. Figs. 4 *b* and 4 *c* illustrate the absorptive capacity of various

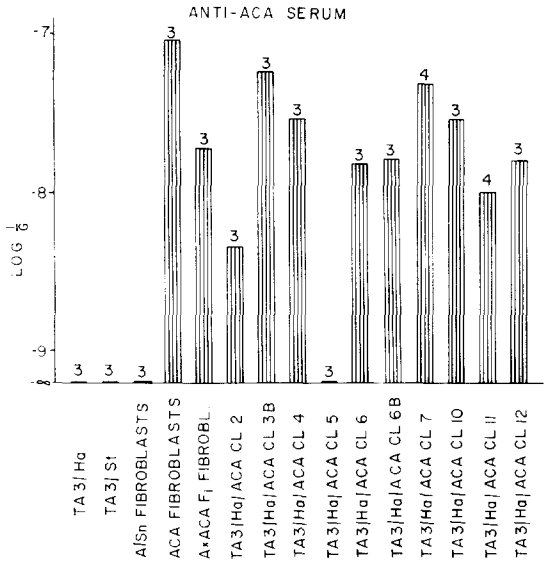


FIG. 5. Absorptive capacity of the TA3 lines, parental and F_1 hybrid fibroblasts, and the TA3/Ha/ACA hybrid clones, tested against the anti-*H-2*^{f} serum.

TA3/Ha/ACA clones. Clones 2, 3B, 4, 7, and 11 had significant absorptive capacity, but clone 5 had no detectable *H-2*^{f} antigen. Fig. 5 shows the 1/*G* values for the *H-2*^{f} antigen system. Each column is based on at least three independent tests. Both TA3 lines lack significant absorptive capacity, as expected. The absorptive capacity of the homozygous ACA and the heterozygous A × ACA fibroblasts differs by a factor of 4.6. This exceeds the reduction in antigen dosage that might be expected to occur in a heterozygote, if antigen expression is determined by an equal contribution from both parental genomes. It has, however, been found previously that sarcomas induced by methylcholanthrene in (A × ACA)F_1 hybrid mice showed considerable asymmetry of antigen expression, with an excess of *H-2*^{a} over *H-2*^{f} antigens (11).

All TA3/Ha/ACA hybrid clones except clone 5 contained significant amounts of ACA-derived *H-2*^{f} antigen. The values fluctuated about the level found in

the (A × ACA)F_1 fibroblasts. Clones 3B and 7 showed somewhat higher levels although they did not reach the levels found in the homozygous ACA fibroblasts. Clones 4, 6, 10, and 12 showed levels close to the heterozygous value, whereas clones 11 and 2, particularly the latter, showed significantly lower values. Clone 5 appeared to contain no ACA-derived antigen. This clone had the highest absorptive capacity for antibody against the *H-2*^{a}–determined *H-2*^{k} and *H-2*^{d} antigens (see Figs. 2 and 3). These two facts may be related. When variant sublines of *H-2*–heterozygous tumors are selected by passage in a parental strain, the disappearance of one *H-2* complex is accompanied by an increase in the concentration of the other, which may reach levels characteristic of homozygous cells (12, 13).

DISCUSSION

The antigenic behavior of the TA3/Ha/ACA hybrids differs sharply from that previously observed in Ehrlich hybrids (1, 2). The diminished expression of the *H-2*^{a} antigens in the TA3/Ha line, which is, in all probability, responsible for the generalized homotransplantability of this tumor, behaves on hybridization as if it were a "recessive" trait. The TA3/Ha cell not only fails to suppress the *H-2*^{f} antigens introduced into the hybrid by the ACA fibroblast, but the expression of its own *H-2*^{a} antigen rises to a level comparable to, or exceeding, that characteristic of the (A × ACA)F_1 hybrid fibroblast. This "release" from antigenic suppression is not dependent on the presence of the *H-2*^{f} complex derived from the ACA fibroblast because in at least one hybrid clone (No. 5) there was no *H-2*^{f} complex, perhaps due to the loss of the corresponding chromosome. The alternative possibility that this clone arose from a TA3/Ha × TA3/Ha homokaryon appears less likely since there is no obvious reason why, in that case, the remarkable rise in *H-2*^{a} antigen concentration should have occurred.

Taken as a whole, our findings suggest that the reduced antigen expression in the TA3/Ha cells is due to some deficiency that can be made good by the ACA fibroblast. Some rate-limiting step in the synthesis of antigen may be involved or the mechanism by which *H-2*^{a} antigens are incorporated into the cell membrane. On the other hand, the membrane structure of the TA3/Ha cell may itself have changed in a way that does not allow full antigen expression. TA3/Ha cells differ from TA3/St in several respects. For example, unlike all other ascites tumors that have been investigated, including TA3/St, the TA3/Ha line cannot be agglutinated by concanavalin A, despite the ability of the cells to bind the lectin.[3]

The mechanism by which the Ehrlich cell suppresses surface antigens must be quite different, for in hybrids between Ehrlich cells and other fully antigenic cells, antigenic suppression is dominant; the antigens contributed by the partner

[3] Friberg, S., Jr., S. H. Golub, B. Lilliehöök, and A. J. Cochran. 1972. Assessment of Concanavalin A reactivity by inhibition of tumor cell migration. *Exp. Cell Res.* In press.

cell, whether *H-2* or virus-determined, are suppressed in the hybrid. Another situation where antigen suppression is recessive in the hybrid cell is the case of the Moloney virus (murine leukemia virus [MLV])[4]–determined surface antigen. The YACIR subline of the YAC lymphoma (14) shows greatly reduced expression of the MLV antigen. When this cell is fused with the A9 fibroblast, full antigen expression is reestablished (15). The antigen suppression in YACIR again differs from that seen in the Ehrlich cell and in hybrids derived from it. In the latter case, both *H-2* and virus-induced surface antigens are suppressed, whereas in YACIR only the MLV-determined surface antigen, and not the *H-2* antigen, is suppressed. In both TA3/Ha and YACIR there is a specific defect in the mechanisms determining the full expression of the surface antigens and this defect disappears when these cells are fused with others in which antigenic expression is normal. In the Ehrlich cell suppression is nonspecific affecting many surface antigens, and it is maintained in the hybrid cell.

Cell hybridization is obviously a useful tool for the study of the factors governing the expression of membrane antigens in mammalian cells and of the types of variation that may be encountered in the expression of these antigens. These variations may be of considerable importance in giving rise to "immunoresistant" tumor sublines capable of escaping immunological rejection reactions.

SUMMARY

The Ehrlich ascites tumor, which has been subjected to prolonged selection for growth in allogeneic hosts, possesses powerful mechanisms for the suppression of antigens normally found on the cell surface. It has previously been shown (1, 2) that when cells in which surface antigens are fully expressed are fused with Ehrlich cells, the suppressive mechanisms of the latter continue to operate and the new surface antigens introduced into the hybrid cell by the other partner are also suppressed. In the present paper we describe the properties of hybrids in which one parent cell was the TA3 ascites carcinoma. There are two sublines of this carcinoma which originally arose as a spontaneous mammary carcinoma in a strain A mouse. The TA3/St line has a high concentration of $H\text{-}2^a$ antigens and shows a strain-specific transplantation behavior; the TA3/Ha subline has a drastically reduced antigen concentration and readily transgresses histoincompatibility barriers. The immunoresistant TA3/Ha subline arose spontaneously without having been subjected to any known immunological selection pressure. Hybridization of TA3/Ha cells with normal diploid ACA fibroblasts reestablished full expression of $H\text{-}2^a$ antigens in nine independently derived hybrid clones. Full reestablishment of both D- and K-end components of the $H\text{-}2^a$ complex could be demonstrated. In some hybrid clones the concentration of $H\text{-}2^a$ antigens was found to be comparable to that seen in $(A \times ACA)F_1$ fibroblasts, whereas in others a higher concentration was observed, even exceeding, in some cases, the levels found in the TA3/St line. The

[4] *Abbreviation used in this paper:* MLV, murine leukemia virus.

H-2^{f} complex, contributed by the ACA parent cell, was fully expressed in eight of the nine hybrid clones studied. Antigen suppression thus behaves as a recessive character in the TA3/Ha hybrids, whereas in the Ehrlich hybrids antigen suppression is dominant.

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