LACK OF DISTINCTIVE SURFACE ANTIGEN ON CELLS TRANSFORMED BY MURINE SARCOMA VIRUS*

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There is a close morphological and biochemical relationship between leukemia and sarcoma viruses of the oncorna type. There is a functional relationship as well, since the leukemia viruses act as helpers, required for the production of infectious sarcoma virus. The genetic information contributed by the sarcoma virus is required, on the other hand, for the induction of solid tumors and the transformation of monolayer cultures. One remarkable characteristic of the murine sarcoma virus is its ability to transgress species barriers. The murine leukemia viruses are essentially confined to their host species and many of them have exacting requirements within the species: their oncogenic effect is strongly dependent on host genetic factors. The murine sarcoma viruses (MSV) have not been distinguishable from the murine leukemia viruses (MuLV) by such characteristics as size (1), kinetics of inactivation (1, 2), morphology (3), or antigenic specificity (4–7). As a rule, however, the immunological studies have been performed with MSV-induced tumors or with in vitro transformed cells that released both MSV and MuLV.

One such study (7) has been carried out with the specific purpose of distinguishing between MSV- and MuLV-associated antigens on MSV-induced sarcoma cells. Extensive cross-absorption of anti-Moloney leukemia virus (MLV) and anti-MSV sera with leukemia and sarcoma cells did not give any evidence of antigens specifically associated with MSV-transformed cells that were not also present on MLV-induced leukemia cells. Furthermore, tolerance to MLV was not broken by MSV inoculation. No virus-specific antigen was detected on the surface of MSV-induced nonproducer hamster cells.

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1 Abbreviations used in this paper: D56-M, MLV superinfected D56 cells; MLV, Moloney leukemia virus; MSV, murine sarcoma virus; MuLV, murine leukemia viruses; NP, nonproducing; S+L−, sarcoma positive, leukemia negative; TATA, tumor-associated transplantation antigens; TSTA, tumor-specific transplantation antigens.

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Recently, some MSV-transformed mouse sarcoma lines have been isolated by appropriate selection procedures that do not produce any helper MuLV nor do they make any MSV (8, 9). Two different types of MSV-transformed 3T3 cells have been described within this category. One is a true nonproducer line (10), isolated from BALB/3T3 monolayers infected with the Moloney isolate of MSV. The other isolate, designated S+L− (sarcoma positive, leukemia negative) was selected from among semisolid agar colonies of MSV-transformed Swiss/3T3 cells (9). Although S+L− cells do not release any infectious virus, they produce a small number of C-type particles that are indistinguishable from infectious murine leukemia-sarcoma virus (11). It was suggested that these particles represent a form of MSV deficient in one or more of the viral components required for infectivity.

In view of the fact that the previous antigenic characterization of MSV (MuLV)-transformed cells was carried out with target cells that produced large quantities of MuLV, the exclusive demonstration of MuLV-type but no MSV-specific surface antigens appeared questionable. Conceivably, the strong MuLV-determined surface antigens may have overshadowed any MSV-specific components. The question was therefore reexamined by the surface antigenic characterization of the S+L− cells reported in the present study.

Materials and Methods

Cells.—The following cell lines were used: (a) normal 3T3 cells from NIH/Swiss mice; (b) S+L− cells isolated from semisolid agar colonies of MSV-transformed 3T3 cells, twice cloned by plating at limiting dilutions in microplates (11); (c) D56 cells, a mixture of S+L− cells and normal cells. It has been shown that murine leukemia viruses induce plaque formation on D56 monolayers and a sensitive titration method has been developed for MuLV on this basis (12); (d) MLV superinfected D56 cells (D56-M). Superinfection was performed as described by Bassin et al. (12). One culture which had 12 plaques on the 5th day was kept in passage. Antigenic conversion, demonstrated by a positive reaction with anti-MLV sera in the mixed hemadsorption test (13), occurred from the 3rd passage onwards; (e) Ha2 line, an MSV-induced tumor of the CBA mouse strain grown in tissue culture. It produces both MSV and MLV (7). Cell lines (a), (b), and (c) have been provided through the courtesy of Dr. R. H. Bassin, National Cancer Institute, Bethesda, Md.

The medium used for growth and maintenance of all cells was McCoy's 5a (modified) supplemented with 10% fetal bovine serum. For the lymphocyte cytotoxicity assay and mixed hemadsorption the cells were grown in Eagle's minimal essential medium (Earle's salts) supplemented with 10% fetal bovine serum (inactivated for 60 min at 56°C). They were seeded in the wells of Microtest plates (Falcon Plastics, Div. B-D Laboratories, Inc., Los Angeles, Calif.) and incubated 18-24 hr at 37°C in 5% CO2 before testing.

Virus.—A 50% homogenate of the YAC, a Moloney virus-induced lymphoma of strain A/Sn origin, served as the source of Moloney virus (MLV) (13). MSV was derived from the 224th passage in BALB/c mice, kindly provided by J. B. Moloney, National Cancer Institute, Bethesda, Md.

Sera.—Anti-D56 serum was produced by inoculating five C57BL mice with D56 cells in doses ranging from 7.0 × 10⁴ to 1.9 × 10⁶ cells twice weekly over a period of 4 months. Serum was collected and pooled during the last month of immunization.

Anti-MLV serum: Collected from 30 (A × C57/leaden)F1 hybrid mice 90-180 days after a single inoculation of a 10% homogenate, prepared from YAC.

Anti-MSV serum: Suspension of a primary MSV-induced tumor of C57BL origin was
irradiated with 10,000 R and inoculated into six C57BL mice. Sera were collected from three tumor-free mice 2 months later, pooled, and designated pool I. The sera of three tumor-bearing mice were designated as pool II. In another series, MSV was inoculated into a group of six adult C57BL mice; all developed tumors that regressed within 1 month. Serum pool III was collected from these mice 1 month after complete regression.

Absorption of C57BL Anti-D56 Serum with Normal 3T3 Cells. —4 × 10^6 normal 3T3 cells were mixed with 0.3 ml of undiluted serum and incubated for 30 min at 37°C, followed by 30 min at 4°C. The cells were removed by one centrifugation at 1500 g. The entire absorption procedure was repeated twice. A final centrifugation at 5000 g concluded the procedure and the serum was frozen and stored at −20°C.

Mixed Hemadsorption Test.—Cells were seeded in the wells of Falcon microtest plates (Falcon Plastics, Div. B-D Laboratories, Inc.) and incubated for 18–40 hr at 37°C in 5% CO₂. A micromodification of the mixed hemadsorption technique was used (14). Veronal-buffered saline containing 5% gelatin was used as diluent and washing fluid. The indicator cells were prepared as previously described (15). Normal mouse serum and all antisera were tested at reciprocal dilutions of 3, 10, 30, 100, 300, and 1000. Six wells with buffer alone were included in each microtiter plate. The test was performed as described previously (15). In all experiments two different combinations of amboceptor and anti-mouse gamma globulin serum were used: 1:1200, 1:50 and 1:800, 1:30, respectively. The strength of the reaction was evaluated under the microscope and graded as follows: +++++, all target cells covered by a heavy layer of sensitized sheep erythrocytes; ++++, almost all target cells show attached erythrocytes, most of them with complete rosettes; ++, 50–100% of the cells show three or more attached erythrocytes, with some complete rosettes; +, less than 50% of the cells have two or more attached erythrocytes; —, no attached erythrocytes.

Lymphocyte Cytotoxicity Assay.—The lymphocyte cytotoxicity assay was carried out by the method of Takasugi and Klein (16). Lymphocytes were harvested from peripheral lymph nodes of (a) BALB/c mice 24 days after MSV infection of 1-month old animals, at the time of tumor regression; (b) Fischer rats, 49 days after MSV infection of 3-day old animals, either at the time of tumor regression (regressor rat) or from a tumor-bearing rat (progressor); and (c) control animals of the same age. The nodes were teased apart in ice-cold medium, mixed thoroughly to break clumps, and passed through a nylon mesh filter. The lymphocytes were washed in serum-free medium by three cycles of centrifugation, finally resuspended in minimal essential medium containing 10% fetal bovine serum, and were applied to the target cells in three concentrations. The relative percentage of surviving target cells was calculated from the mean of the six replicates receiving immune lymphocytes, compared with the control wells with the same ratio of normal lymphocytes and target cells.

RESULTS

Serology.—Two different approaches were used to search for possible MSV-specific surface antigens, as shown in Table I. In one approach, isoantibody-free sera were obtained by immunization with MLV of syngeneic origin or by allowing primary, autologous MSV sarcomas to regress. The sera were subsequently tested for reactivity against MSV-induced sarcoma cells. In the second approach, alloantisera obtained by immunizing C57BL mice with D56 cells were absorbed with the corresponding nontransformed 3T3 cells. After all activity against 3T3 had been removed, the sera were tested against D56 cells.

None of the two MSV-transformed, MuLV-free cell lines (S+L− and D56) gave any reaction with the anti-MLV and anti-MSV sera. Both types of sera reacted well with the MLV-superinfected D56-M line and with the MSV (MLV)
### TABLE I

Reactivity of MSV-Transformed Cell Lines in Mixed Hemadsorption Test

| Reciprocal dilution of antiserum | Cell lines | Normal 3T3 | S + L-- | D56 | D56-M | Ha2 |
|----------------------------------|------------|------------|--------|-----|-------|-----|
| No. of tests                     |            |            |        |     |       |     |
|                                  |            | 6          | 6      | 10  | 8     | 2   |
| **MLV***                         |            |            |        |     |       |     |
| 3                                |            | --         | --     | --  | --    | ++  |
| 10                               |            | --         | --     | --  | ++    | +   |
| 30                               |            | --         | --     | --  | +     | --  |
| 100                              |            | --         | --     | --  | --    | --  |
| **MSV I**                        |            |            |        |     |       |     |
| 3                                |            | --         | --     | ++  | +     |
| 10                               |            | --         | --     | ++  | +     |
| 30                               |            | --         | --     | +   | +     |
| 100                              |            | --         | --     | --  | --    | --  |
| **MSV II†**                      |            |            |        |     |       |     |
| 3                                |            | --         | --     | +++ | ++    |
| 10                               |            | --         | --     | ++  | +     |
| 30                               |            | --         | --     | +   | ++    |
| 100                              |            | --         | --     | +   | +     |
| 300                              |            | --         | --     | --  | +     |
| **MSV III§**                     |            |            |        |     |       |     |
| 3                                |            | --         | --     | +++ | +     |
| 10                               |            | --         | --     | ++  | +     |
| 30                               |            | --         | --     | +   | ++    |
| 100                              |            | --         | --     | +   | +     |
| 300                              |            | --         | --     | --  | +     |
| 1000                             |            | --         | --     | --  | --    |     |
| **C57BL anti-D56**               |            |            |        |     |       |     |
| 3                                |            | +++        | ++     | ++  | +++   |
| 10                               |            | ++         | ++     | ++  | ++    |
| 30                               |            | ++         | ++     | ++  | +     |
| 100                              |            | +          | +      | +   | +     |
| 300                              |            | +          | --     | +   | +     |
| 1000                             |            | --         | --     | +   | +     |
| **C57BL anti-D56 absorbed**      |            |            |        |     |       |     |
| 3T3                              |            | --         | --     | --  | --    |
| 10                               |            | --         | --     | --  | --    |
| 30                               |            | --         | --     | --  | --    |

Evaluation of the strength of the mixed hemadsorption reaction: ++++, almost all target cells show attached erythrocytes, most in complete rosettes; ++, 50-100% of the cells show three or more attached erythrocytes, some complete rosettes; +, less than 50% of the cells have two or more erythrocytes sticking to their surface; --, no erythrocytes.

* Serum collected from mice immunized with irradiated tumor cells.
† Serum collected from tumor-bearing mice.
§ Serum collected from postrejection hosts.
producing Ha2. It is of interest to note that the sera of mice that rejected their own primary MSV-induced tumors gave the highest antibody titers (1:300) against the D56-M cells. The serum of tumor-bearing animals (MSV II) gave a somewhat lower titer (1:100). Animals pretreated with irradiated leukemia (MLV) or sarcoma (MSV I) cells titered 1:30 against D56-M cells.

Normal 3T3, S+-L--, and D56 cells were all highly reactive with the C57BL anti-D56 serum. This was, however, due to antibodies against the histocompatibility antigens of D56 cells recognized by the C57BL mice, since it could be removed by absorption with normal 3T3 cells.

These experiments suggest that S+-L-- cells, although they contain the MSV genome, do not differ from the original 3T3 cells in their antigenic characteristics.

Cell-Mediated Cytotoxicity.—Table II shows the effect of mouse or rat lymphocytes on D56 and Ha2 cells, respectively. Immune lymph node cells of both kinds reduced the number of Ha2 cells but not the number of D56 cells per well. Ha2 cells were killed by mouse lymph node cells most notably on the 24th day after infection, about 10 days after tumor regression. In line with the

### Table II

| Donor of lymph node cells* | Lymph node cell: target cell ratio | 100:1 | 50:1 | 25:1 |
|---------------------------|-----------------------------------|-------|------|------|
|                           | No. of cells/ well ± SD | %    | No. of cells/ well ± SD | %    | No. of cells/ well ± SD | %    |
|                           | Control | MSV infected | Control | MSV infected | Control | MSV infected | Control | MSV infected |
| Regressor mouse           |         |               |         |               |         |               |         |               |
| Ha2                       | 119 ± 23 | 41 ± 21      | 31     | 180 ± 36 | 75 ± 38 | 41     | 137 ± 55 | 80 ± 30 | 33±  |
| D56                       | 132 ± 27 | 153 ± 40     | 116    | 86 ± 39  | 144 ± 41 | 168    | 131 ± 27 | 172 ± 24 | 13±  |
| D56-M                     | 167 ± 42 | 98 ± 38      | 199    | 164 ± 57 | 145 ± 82 | 89     | 130 ± 42 | 160 ± 69 | 16±  |
| Regressor rat             |         |               |         |               |         |               |         |               |
| A78                       | 132 ± 11 | 75 ± 8       | 37±  | 186 ± 14 | 144 ± 21 | 77±  | 152 ± 17 | 172 ± 23 | 11±  |
| Ha2                       | 312 ± 18 | 221 ± 6      | 77±   | 309 ± 17 | 275 ± 12 | 72±   | 327 ± 20 | 299 ± 15 | 9±   |
| D56                       | 162 ± 19 | 329 ± 36     | 203    | 172 ± 18 | 180 ± 31 | 104    | 197 ± 86 | 253 ± 30 | 122   |
| Regressor rat             |         |               |         |               |         |               |         |               |
| A78                       | 191 ± 23 | 121 ± 15     | 61±  | 144 ± 19 | 127 ± 10 | 88±  | 141 ± 3  | 99 ± 21 | 17±  |
| Ha2                       | 200 ± 7  | 131 ± 11     | 66±   | 205 ± 9  | 151 ± 7  | 73±   | 193 ± 21 | 208 ± 11 | 118   |
| D56                       | 68 ± 9   | 68 ± 12      | 100    | 76 ± 32  | 129 ± 12 | 163    | 61 ± 14  | 137 ± 6  | 25±  |

P values: 1 < 0.0005, 2 < 0.005, 3 < 0.025.

* Regressor mouse: lymph node cells were harvested from BALB/c mice 24 days after MSV infection at the time of tumor regression. Rat: lymph node cells were harvested from Fischer rats 49 days after MSV infection, either at the time of tumor regression ( regressor) or from a tumor-bearing rat ( progressor).

† Relative percentage of surviving target cells was calculated as follows:

   Mean No. of target cells in six replicate wells receiving immune lymphocytes × 100.
   Mean No. of target cells in six replicate wells receiving normal lymphocytes

The cytotoxicity of immune lymphocytes was compared with that of normal lymphocytes.

Table II shows the effect of mouse or rat lymphocytes on D56 and Ha2 cells, respectively. Immune lymph node cells of both kinds reduced the number of Ha2 cells but not the number of D56 cells per well. Ha2 cells were killed by mouse lymph node cells most notably on the 24th day after infection, about 10 days after tumor regression. In line with the
results of the mixed hemadsorption test, where a new, virus-associated antigen could be detected on D56 cells after superinfection with MLV, immune lymphocytes killed the D56-M cells, while the same lymphocytes did not affect D56 cells. Rat lymph node cells harvested from tumor-bearing rats (progressor) or from rats after tumor regression (regressor) were equally cytotoxic for Ha2 cells. A similar cytotoxic effect could be demonstrated on A78 cells (an MLV-MSV producer cell line of Wistar rat origin). When the same lymphocytes were tested against D56 cells they did not reduce the target cell number, however, but rather stimulated target cell growth. Control lymphocytes produced no cytotoxicity against either line.

**DISCUSSION**

The present study thus confirms the previous evidence, obtained with double producing cells, showing that there is no evidence for any MSV-specific surface antigen. No antibodies directed against S+L− or D56 cells could be detected in the sera of mice immunized with MSV or with MSV-induced tumor cells and immune lymphocytes also failed to give any evidence of MSV-specific cytotoxicity. It is therefore difficult to avoid the conclusion that, unlike other oncogenic and transforming viruses, MSV does not induce the appearance of new surface antigens in the transformant target cell, known to carry the viral genome.

An analogous conclusion was obtained by Stephenson and Aaronson (17), using a different approach. Nonproducing (NP) MSV-transformed BALB/3T3 cells were found to grow equally well in virus-immunized and control mice and thus lacked a tumor-specific transplantation antigen. In contrast to the S+L− cells used in the present study, NP cells were negative for virus particles by electron microscopy or by the uridine-3H incorporation method. In addition to the production of noninfectious particles (10), as already mentioned, S+L− cells also differ from NP cells by containing at least some of the MuLV gs antigens (18) that are absent from NP cells (10). Superinfection of S+L− cells with MuLV induces plaque-like lesions, furthermore, while nonproducer cells do not show any apparent morphological changes. It therefore seems that S+L− cells express more virally associated genetic information than nonproducer cells. While sufficient for the production of noninfectious and presumably incomplete virus particles and the synthesis of gs antigens, this information apparently does not include the mechanism required for the induction of virus-associated cell membrane antigen(s). It may be noted also that the noninfectious particles are also deficient with regard to at least one other property that is a regular feature of C-type viruses: they contain no reverse transcriptase (19).

It can therefore be stated that (a) MSV-transformed cells do not carry a *sui generis* virus-specific membrane antigen; the presence of an MLV-type surface antigen signals infection with leukemia virus. (b) Surface changes de-
tectable as antigens do not necessarily have to accompany viral oncogenesis in all its forms.

Tumor-specific transplantation antigens (TSTA) or, more appropriately, tumor-associated transplantation antigens (TATA), have been found in all virus-induced tumors that have been studied previously (20-22). They are group specific in the sense that the same or overlapping antigenic specificities can be demonstrated in all tumors induced by the same virus, even if they arise in different tissues and different species, but are different in tumors induced by unrelated viruses. In view of the fact that such antigens, e.g. the polyoma-associated TSTA, were resistant to prolonged negative selection against antigenic cells by passage in preimmunized host (23), together with the fact that their presence (and the presence of other virally induced transplantation antigens as well) was regularly associated with neoplastic transformation, it was speculated (20, 21, 24) that the rearrangement of the outer cell membrane reflected in the appearance of the new antigens may represent the key change that makes the cell disobedient to growth control, i.e., neoplastic. This idea was particularly attractive as far as the small oncogenic DNA viruses are concerned, in view of their very limited amount of genetic information.

The absence of any detectable virally associated surface antigen on MSV-transformed cells in the present study, together with the lack of any transplantation antigen in the experiments of Stephenson and Aaronson (17) raises doubts about the validity of this theory; or at least its general applicability to all virus-induced systems. Conceivably, MSV-induced cell transformation may still be accompanied by important membrane changes that are not recognized by the animal as new, foreign antigens, or not sufficiently to be detected by the methods used. Alternatively, neoplastic growth may not be due, in this case at least, to changes at the level of growth-regulating membrane receptors, but may reside at some more central level within the cells.

In addition to the MSV system, TSTA-like antigens were also undetectable on a minority of chemically induced tumors in the rat (25). Moreover, hybrid cell lines derived from the fusion of polyoma virus-induced mouse tumor cells and mouse L cells (A9 subline) that showed a suppression of malignant behavior still carried the polyoma transplantation antigen (26), indicating that the presence of the antigen is not sufficient for the expression of a malignant potential. In the C-type leukemia virus systems, the virally determined surface antigen, carried by the leukemic cells, can also be demonstrated, apparently with the same specificity, on functionally unaffected lymphocytes in virus-infected hosts (27) or in apparently unaltered normal bone marrow-derived culture lines exposed to the leukemia virus (28).

**SUMMARY**

Some murine sarcoma virus (MSV)-transformed mouse 3T3 cells contain the MSV genome in the absence of infectious helper murine leukemia virus (MuLV) and MSV production.
These cells, designated S+L− (sarcoma positive, leukemia negative), were analyzed for the presence of a possible MSV-determined membrane antigen by the mixed hemadsorption test and in vitro lymphocyte cytotoxicity assay. Two different serological approaches were used: (a) isoantibody-free sera were obtained by immunizing with MSV of syngeneic origin or by allowing primary, autologous MSV sarcomas to regress, or (b) alloantisera obtained by immunizing C57BL mice with S+L− cells were absorbed with the corresponding nontransformed 3T3 cells until all activity against 3T3 had been removed.

While MuLV-superinfected S+L− cells and a culture line of an MSV sarcoma known to produce both MSV and MLV were highly reactive, normal 3T3 and S+L− cells were negative. Similarly, lymph node cells from MSV immune mice or rats did not kill S+L− cells, although they were cytotoxic against target cells known to carry MuLV-associated antigens. Thus, the present study gives no positive evidence for the existence of any MSV-induced new surface antigen in the transformed target cell, known to carry the viral genome.

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