RELATIONSHIP BETWEEN ANTIGENICITY AND MORPHOLOGY OF MURINE LUNG ADENOMATA

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Summary.—Thirty-six lung adenomata induced in mice by urethane followed or not by cortisone, all had an adenomatous morphology at the first s.c. transplant in syngeneic hosts. Seventeen of them acquired a sarcomatous structure within a few s.c. transplant generations whilst the other 19 remained adenomatous for as long as tested, i.e. at least 10 transplant generations. The change of morphology was not dependent on s.c. growth, since tumours also transformed when allowed to grow in the lung, and was not correlated to the capacity of a tumour for growth or metastasis. The 2 types of tumours were antigenically different, since only tumours that after few transplants changed their morphology were found at the first s.c. transplant to possess tumour-associated membrane antigens as revealed by an in vitro test. In addition, only the tumours which acquired a sarcomatous morphology were found gs-positive. The majority of antigenic primary tumours arose in mice belonging to the groups of treatment which induced the strongest immunodepression. It is suggested that a predisposition to sarcoma progression is related to an immunological control, at the time of adenoma induction, of an oncornavirus, responsible for the superimposed sarcomatous change.

Spontaneous or chemically induced primary murine lung adenomata are histologically similar, having a glandular pattern with cells arranged in acini and often disposed in papillary formations. During successive transplant generations in syngeneic hosts the tumours grow progressively faster and some of them change from the adenomatous to a sarcomatous pattern (Stewart et al., 1947; Stewart, 1959). C-type virus particles for which no oncogenic role in lung adenomagenesis has been demonstrated, have been observed in spontaneous or chemically induced murine lung adenomata and been seen to increase during transplantation (Bucciarelli, 1971; Bucciarelli and Ribacchi, 1972; Kimura et al., 1972).

The aim of the present experiment was to establish whether the change in morphological structure of urethane-induced lung adenomata during transplantation is correlated with certain tumour characteristics such as malignancy, evaluated as capacity for growth or metastasis, or presence of tumour-associated cell-surface antigens or viral antigens. Having previously demonstrated (Colnaghi et al., 1971; Ménard et al., 1973) that lung adenoma antigenicity was directly correlated with the treatment-induced immunological impairment of the primary host, we now report on the influence of this impairment on the proneness of the morphological structure to modify during transplantation.

MATERIALS AND METHODS

Animals.—Inbred BALB/c mice and the (C3Hf × BALB/c)F1 hybrids of both sexes, maintained in this laboratory, were used.

Tumours.—Lung adenomata were induced by 5 i.p. injections of urethane 0.2 or 1 mg/g body weight once every second day in BALB/c mice, starting at 10 days of age (Group A and B). Two other groups
of mice (C and D) received the same urethane
treatments and in addition, on alternate
days, 5 injections of 0·1 mg/g body weight of
cortisone. In a previous paper, it was
reported that the 4 types of treatment caused
an increasing immunological impairment,
evaluated by the Jerne technique, from
Group A to D, and that the antigenicity
of the lung adenomata was inversely related
to the immunodepression of the tumour host
(Ménard et al., 1973). The mice were
killed when they showed dyspnoea, the
adenomatous nodules were dissected from
the normal tissue and a cell suspension was
mechanically prepared in Hanks' balanced
salt solution (HBSS). The cells were then
used for in vitro transplantation or were
grown in vitro for the cytotoxicity test.

In vivo studies.—The tumours were
maintained in vivo by s.c. or i.v. inoculation
of 0·2 ml of the cell suspension, containing
about 1 x 10^6 cells, prepared from the
primary lung adenomata or from the s.c.
transplants or from pulmonary nodules.
The s.c. inoculum was into the right flank
and the i.v. into the tail vein of syngeneic
mice of the same sex as the tumour donor.

The animals were killed for tumour
morphology examination and subsequent
transplantation when the tumours reached
10 x 10 mm in diameter or when, after the
i.v. inoculation, mice presented dyspnoea.

Cytotoxicity test.—The cell suspension,
obtained as described, was centrifuged and
the cells were suspended in medium 199
supplemented with 20% foetal calf serum,
100 i.u./ml penicillin and 50 µg/ml strepto-
mycin. The cells were seeded directly in
tissue culture plastic microplates (No. 3034
Falcon Plastics, Los Angeles, Calif.) with
60 wells of 10 µl each, into which were
delivered 500 viable tumour cells in 10 µl
medium. The microplates were incubated
at 37°C in a 5% CO₂ humidified atmosphere
and 4 days later the medium was renewed
and the plates were incubated for a further
3 days. Then the microplates were washed
and 5 x 10^4 viable lymphocytes in 10 µl
medium were delivered into each well in
which 300–500 tumour target cells were
found. After 48 h incubation the micro-
plates were washed carefully with HBSS
to remove lymphoid and dead cells and
viable attached cells were fixed in methanol
and stained with May-Grunwald-Giemsa.
Counting was carried out under the micro-
scope with the help of a 25-square grid
covering the entire floor of the well except
the margins, and taking into consideration
only epithelial-like cells present in 10 squares
per well. The significance of the difference
between the adenoma cell number after
exposure to experimental or control effector
cells was evaluated by Student's t test.
Differences were considered significant when
P was 0·01 or less.

Lymphocytes.—2–3 month old BALB/c
mice of the same sex as the tumour donors
were injected s.c. with 0·2 ml HBSS contain-
ing 1 x 10^6 living cells from the cell
suspension of BALB/c transplanted adenoma.
The subcutaneous tuumours, grown
about 10 mm in diameter, were surgically
removed and 10 days afterwards effector
cells were obtained by purification with
Ficoll-Triosil method from spleen of tumour-
or sham-operated animals.

Determination of the gs antigen.—Cell
suspensions in HBSS from transplanted
lung adenomata were prepared. The cells
were packed by centrifugation, resuspended
in distilled water for 20 min, homogenized
at 4°C at about 14,000 rev/min and centri-
fuged at 100,000 g for 1 h. The supernatants
were preserved and the pellets were
added with 20 volumes of peroxide-free
ether and shaken for 2 h. The ether was
then removed by cold N₂ and the ether-
treated extracts were mixed each with its
own supernatant and centrifuged at 100,000 g
for 1 h. The pellets were discarded, and
the supernatants dialysed against distilled
water, lyophilized, resuspended in a volume
of HBSS reduced to approximately one-
fifth the initial tissue weight and then tested
in doubling dilutions by the double diffusion
test with a rat antiserum having a precipi-
titating activity for gs antigen.

Histology.—Fragments of primary and
transplanted lung adenomata were prepared
for light microscopy, the tissues being
fixed in Bouin solution. Paraffin sections
were stained by haematoxylin and eosin.

RESULTS

Changes in morphology

Thirty-six BALB/c primary lung ade-
omata, equally distributed for their
origin from the four treatment groups,
and their successive transplants, were
All the primary tumours presented an adenomatous structure and grew slowly when transplanted s.c. into a syngeneic host, reaching a size of about 10 × 10 mm diameter in a mean time of 112 ± 10 days. Often the tumour-bearing animals were still alive 1 year after inoculation. During the subsequent transplants all the tumours grew progressively faster: 17 of them changed into sarcomata by the 5th transplant, whereas the other 19 retained their initial adenomatous morphology at the 10th transplant generation (Fig. 1). In most instances the morphological modification was abrupt, although on several occasions admixtures of glandular structures and a solid growth resembling sarcoma were observed (Fig. 2, 3 and 4). Five of the 19 tumours which were found to retain the adenomatous morphology at the 10th passage, were studied also at subsequent transplants and no changes in morphology were noted (Fig. 5).

To exclude the possibility that the sarcomatous change was due to a recruitment of mesenchymal host cells from the fibrous capsule surrounding tumours, a primary adenoma from the most immunodepressed group of treatment (D)
Fig. 3.—A transition form between adenomatous and sarcomatous morphology at the 2nd s.c. transplant. × 375.

Fig. 4.—Sarcomatous structure of a lung adenoma at the 8th s.c. transplant. × 375.
was serially transplanted s.c. in hybrid (C3Hf × BALB/c)F₁ mice, where at the 3rd transplant generation it acquired a sarcomatous structure. The tumour was retransferred into the 2 parental strains, C3Hf and BALB/c, at the 1st, 3rd, and 5th transplant, and was found to grow in BALB/c mice only (Table I).

To rule out a possible role of the subcutaneous location of the tumour transplants, another primary adenoma of Group D was serially injected as well into the tail vein so that the adenomatous cells could reach the lung and grow in their original milieu. The tumour acquired a sarcomatous morphology with both techniques, at the 3rd passage when transplanted s.c. and at the 4th passage subcutaneous injection.

**Table I.**—Growth of a BALB/c Lung Adenoma in (C3Hf × BALB/c)F₁ Hybrids and Subsequent Transfer in the Parental Mice

| Transplant generation | No. of days to a 10 × 10 mm tumour | Tumour take | Morphology |
|------------------------|-----------------------------------|-------------|------------|
| 1                      | 4/4                               | 40          | adenoma    |
| 2                      | 2/3                               | 47          | adenoma    |
| 3                      | 4/4                               | 25          | sarcoma    |
| 4                      | 4/4                               | 20          | sarcoma    |
| 5                      | 4/4                               | 19          | sarcoma    |

| Transfer in | No. of days to a 10 × 10 mm tumour | Tumour take | Morphology |
|-------------|-----------------------------------|-------------|------------|
| C3Hf        | 0/4                               | 42          | adenoma    |
| BALB/c      | 0/4                               | 42          | adenoma    |

* No. of mice with tumour/No. of transplanted mice.
† Mean value for the growing tumours.
‡ Not tested.
when growing in the lung. The tumour cells injected i.v. originated numerous small nodules in all lobes.

*Growth and metastasis characteristics*

The subcutaneous growth of 5 lung adenomata which did not change their morphology after serial passages and of 8 which transformed into sarcomata, was evaluated from the 1st through the 8th transplant generation. As shown in Fig. 6, tumour growth, expressed as the time taken to reach a 10 × 10 mm diameter, was accelerated in the successive transplants for both tumour types, though the sarcomatous tumours grew more rapidly than the adenomatous ones.

Six tumours, 3 adenomatous and 3 sarcomatous, were also studied at various transplant generations for their ability to give rise to lung metastases, within 2, 4, 6 or 8 weeks after s.c. implantation. The mice came under observation either on spontaneous death or on sacrifice when moribund or at the time scheduled for killing. Since no differences were noted at the different transplants, the data for each tumour were pooled (Table II). Two sarcomatous tumours killed the host before the 6th or the 8th week with only 1 case of lung metastases, death being ascribed to the huge s.c. growth. One sarcomatous and 2 adenomatous tumours gave pulmonary metastases, the former earlier and at a higher rate than the latter. The animals implanted with the 3rd adenomatous tumour did not develop metastases.

*Correlation between antigenicity and subsequent morphology*

Nine lung adenomata were tested at the first transplant, when all were adenomatous, for membrane antigenicity by an in vitro microtest for cell-mediated immunity. Five tumours were destroyed by lymphocytes immune against a syngeneic lung adenoma previously demonstrated immunogenic, and all of them acquired the sarcomatous morphology during the subsequent transplants. Four tumours were negative for membrane antigenicity and all of them maintained the adenomatous appearance permanently (Table III). Eleven tumours were tested by immunodiffusion for the presence of

*Table II.—Capability of Adenomatous and Sarcomatous s.c. Tumours to Give Rise to Lung Metastases*

| No. of mice | Tumour type | Treatment group | Transplant generation | Animals with lung metastases/Total |
|-------------|-------------|-----------------|-----------------------|-----------------------------------|
| 42          | sarcoma     | C               | 8-15                  | 0/5 2/18 17/19 no survivors       |
| 32          | sarcoma     | C               | 10-20                 | 0/12 1/20 no survivors no survivors |
| 36          | sarcoma     | B               | 9-17                  | 0/4 0/26 0/6 no survivors         |
| 38          | sarcoma     | B               | 6-12                  | NT* 0/14 0/10 2/14                |
| 37          | adenoma     | A               | 12-17                 | 0/3 0/16 0/9 0/9 0/9              |
| 29          | adenoma     | C               | 14-17                 | NT* 0/10 0/9 3/10                 |

*Not tested.*

*Fig. 6.—Relationship between morphology and growth rate of lung tumours during s.c. transplantation: □□ □ adenomatous, □□ □ sarcomatous tumours.*
TABLE III.—Relationship Between Morphology and Antigenicity of 12 Lung Adenomata

| Tumours | Treatment group | Final morphology | Immune | Normal | % reduction | gs antigen |
|---------|----------------|------------------|--------|--------|-------------|------------|
| Ad 1    | D              | sarcoma          | 38 ± 9 | 74 ± 13| 49†         | +          |
| Ad 14   | C              | sarcoma          | 134 ± 9| 213 ± 12| 38†        | +          |
| Ad 18   | C              | sarcoma          | NT     | NT     |             | +          |
| Ad 24   | A              | sarcoma          | 122 ± 10| 187 ± 13| 35†        | NT‡        |
| Ad 26   | B              | sarcoma          | NT     | NT     |             | +          |
| Ad 44   | B              | sarcoma          | 55 ± 6 | 114 ± 19| 52†        | +          |
| Ad 62   | D              | sarcoma          | 114 ± 8| 224 ± 11| 49†        | +          |
| Ad 29   | B              | adenoma          | 93 ± 13| 87 ± 10 | + 7        | +          |
| Ad 35   | A              | adenoma          | 124 ± 16| 137 ± 12| 10         | +          |
| Ad 36   | A              | adenoma          | 32 ± 5 | 36 ± 6 | 12         | +          |
| Ad 50   | B              | adenoma          | NT     | NT     |             | +          |
| Ad 54   | C              | adenoma          | 116 ± 9| 122 ± 9 | 5          | +          |

* The cytotoxicity test was done on tumours at the first transplant, the gs test on transplanted tumours when they had reached the definitive morphology.
† P < 0.05.
‡ Not tested.

the gs antigen when they had acquired the final morphology; 5 out of 6 sarcomatous tumours tested were positive at dilutions ranging between 1:32 and 1:128 whereas all 5 adenomatous ones were negative, even when tested undiluted (Table III).

Correlation between immunological status of the primary host and subsequent morphology

The 36 lung adenomata used in the present study were originally induced in 4 groups of mice with 4 different carcinogenic schedules of treatment which had a different immunodepressive effect, and their antigenicity as primary tumours correlated directly with the immune status of the host, i.e. the more immunodepressed hosts originated the more antigenic tumours (Colnaghi et al., 1971; Ménard et al., 1973). As shown in Fig. 7, we now report a correlation between the immunological status of the original host and the proneness of the tumours to change morphology, since almost all the tumours that arose in the most immunodepressed group became sarcomatous, whereas about 75% of the tumours arising in the less immunodepressed group remained adenomatous.

DISCUSSION

The change of morphology of lung adenomata during successive transplant generations is an already reported but unexplained phenomenon, occurring in about 50% of the transplanted tumours (Stewart, 1959). We studied various parameters of this phenomenon and the results suggest that the progression from a glandular to a sarcomatous pattern is not a random but a predetermined event. All the lung tumours studied were
adenomatous at the first transplant when they grew slowly, in general without killing the host. We found that, upon serial transplantation, some tumours changed their histologic morphology within a few transplants, becoming sarcomatous, whilst others retained their original adenomatous structure for as long as tested. The change of morphology does not seem to be influenced by the s.c. growth of the transplanted tumours, since the adenomata which eventually became sarcomatous after s.c. transplants, transformed also when allowed to grow in the lung. We can exclude a recruitment of mesenchymal host cells since a BALB/c lung adenoma transplanted in F₁ hybrids and then retransferred in the 2 parental strains, still grew well in the BALB/c host.

The difference in morphology was only partially correlated with the capacity of the tumours for growth or metastasis assumed as indicators of malignancy. The 2 morphologically different types of lung tumours in fact both increased their growth capacity in the successive transplants, although the sarcomatous tumours grew more rapidly than the adenomatous ones. Both types had the capacity to metastasize but the sarcomatous tumours metastasized more rapidly.

The immunological behaviour of the 2 types of tumour differed, since only tumours that changed their morphology after the first few transplants were found to possess tumour-associated membrane antigens at the first transplant. This antigenicity was conditioned by the immune responsiveness of the primary tumour host, since the majority of the antigenic adenomata were observed in the most immunodepressed groups, and seems to be related to a C-type oncornavirus, since a mouse anti-G serum was completely absorbed by antigenic adenomata (unpublished results). Moreover, only sarcomatous tumours were gs-positive, at least as revealed by the sensitivity of the assay we used.

It therefore appears that adenomata destined to become sarcomatous are somehow related to a viral activity. A- and C-type particles have been described in primary lung adenomata and it has been reported that A-particles disappeared upon transplantation whereas C-particles increased (Buccioneri and Ribacchi, 1972). We suggest that a predisposition to sarcoma progression is related to a complex immunological control at the time of adenoma induction that conditions the activity of an oncorna virus which, although perhaps without an etiological role in adenomagenesis, is nonetheless responsible for the superimposed sarcomatous change of tumours occurring in immunodepressed animals. The change does not take place when the immune system of the primary tumour host can control the virus, either by modulating its antigenic expression, as already reported for cellular and viral antigens (Boyse et al., 1967; Ioachim et al., 1972), or by eliminating the antigenic cells.

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