HUMAN SARCOMAS IN CULTURE

FOCI OF ALTERED CELLS AND A COMMON ANTIGEN; INDUCTION OF FOCI AND ANTIGEN IN HUMAN FIBROBLAST CULTURES BY FILTRATES*

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A major achievement of cancer research in animals has been the demonstration that several tumors of natural occurrence are caused by viruses.

One of the foremost goals of human cancer research is now to identify viruses that may be responsible for any form of cancer in man. Two groups of viruses have so far emerged as important in the natural etiology of animal tumors: (a) DNA viruses of herpes-type, responsible for the Lucké adenocarcinoma of frogs (1) and Marek's neurolymphomatosis of chickens (2), and (b) the RNA oncogenic viruses (oncornaviruses) responsible for leukemias and sarcomas of the chicken, mouse, and cat (4, 5).

Among human cancers there is evidence associating a herpes-type virus with Burkitt's lymphoma and nasopharyngeal carcinoma, including reports that it may induce transformation of human leukocytes in vitro (6, 7). On the other hand, because of the many known examples of sarcomas and leukemias induced by oncornaviruses in animals, evidence has been sought for a similar causative virus in man. Virus of this type is often demonstrable by electron microscopy in sarcomas and leukemias of the chicken, mouse, and cat, but this has not been the case with comparable human tumors and leukemias. In man, particles with the morphology of oncornaviruses have only seldom been demonstrable and then usually in numbers so small as to raise questions about their significance (8). Nevertheless this is not strong evidence against participation of oncornaviruses in human malignancy, because many animal tumors which unquestionably are of viral origin, and which carry viral genomes, do not produce complete virus particles. For these reasons, the relatively negative morphological evidence is inconclusive.

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1 Oncornaviruses: a designation recently proposed for this group of viruses (3).
In the case of human sarcomas, more positive evidence has been obtained by other methods. Finkel observed a small yield of osteosarcomas in hamsters inoculated with extracts of human sarcomas (9), and Morton and colleagues have provided substantial immunological evidence of viral etiology (10, 11). This evidence includes the finding of a common antigen in a variety of human sarcomas and the presence of the corresponding antibody (demonstrable by immunofluorescence and complement fixation) in human serum, particularly in relatives and contacts of patients. These studies suggest viral etiology by implying infective transmission, and because cross-reacting antigenicity is characteristic of tumors induced by the same oncogenic virus (12). Morton and colleagues also report that particles resembling oncorna-viruses were observed in foci of distinctive morphology which appeared transiently in a cultured liposarcoma (13), and that the common antigen was induced in cultures of normal human cells by cell-free supernates of positive sarcoma cultures (14). This antigenic conversion (15) was not associated with focus formation, except in one case of an osteosarcoma during its early history in vitro; filtered medium from this culture line induced foci in human diploid fibroblasts, but for unknown reasons neither later cultures of this osteosarcoma nor cultures of any other sarcomas had focus-inducing activity.

The following report is based on a study of 18 sarcomas obtained from patients at Memorial Hospital and maintained in long-term culture

**Materials and Methods**

_Establishment of Sarcoma Lines in Culture._—Sterile specimens of tumor were finely minced with curved scissors, and cell suspensions were prepared by incubation at room temperature for 30–60 min in Puck's saline A medium with 0.25% trypsin (Difco Laboratories, Inc., Detroit, Mich.), 0.002% DNase (Worthington Biochemical Corp., Freehold, N.J.), and 0.02% collagenase (Worthington). The cells were washed in Earle's balanced salt solution and resuspended in Eagle's minimal essential medium (MEM) or Dulbecco's medium containing 200 unit/ml penicillin and 200 μg/ml streptomycin, supplemented with calf serum, fetal calf serum (FCS), horse serum, or human serum (see Text). T-30 flasks were seeded with 0.5 to 1 × 10⁶ cells/ml. For subculture, the cells were freed by trypsin (0.25%), washed, and reseded at a concentration of 2.5 to 5 × 10⁵ cells/ml.

_Other Culture Lines of Human Cells._—

I. Fibroblast lines: (a) WI-38 embryonic lung (16), and (b) embryonic skin (skin fibroblasts [SF]); both from Microbiological Associates, Inc., Bethesda, Md.

II. Epithelial lines (growing as attached cultures): (a) RPMI 4445 amelanotic melanoma; (b) RPMI 5966 melanoma (17); (c) Lev-II breast carcinoma, line established from cells in pleural fluid; (d) BT 20 breast carcinoma (18); (e) AIAb breast carcinoma, lung metastasis (19); (f) Rop renal adenocarcinoma, lung metastasis; and (g) HeLa cervix carcinoma (20).

III. Nonadherent cell lines (not attaching to culture vessel): (a) SK-L1 to 15, 15 different lines established from blood or lymph nodes of patients with leukemia or lymphoma (21);

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* Abbreviations used in this paper: FCS, fetal calf serum; IF, immunofluorescence; MEM, Eagle's minimal essential medium; FBS, phosphate-buffered saline; SF, skin fibroblasts.

* G. Moore and R. E. Gerner. Unpublished data.

* Cell lines established in culture in this laboratory by E. Beth and L. J. Old in 1967. Unpublished data.
(b) SK-RCS1 reticulum cell sarcoma (22); (c) F3HR-I Burkitt's lymphoma (23); (d) SK-Mel 1 melanoma (24); (e) Lev-III, breast carcinoma, nonattaching culture derived from Lev-II (above); (f) Sal-III breast carcinoma line established from cells in ascites fluid (4).

The fibroblastic and epithelial lines (I and II above) were maintained in MEM with 15% FCS and the nonattaching lines (III above) in RPMI 1640 with 15% FCS.

**Immunofluorescence (IF).** Cells from attaching culture lines were trypsinized, washed, and resuspended in MEM with 15% FCS. Drops containing $3 \times 10^4$ cells/ml were placed on glass slides 0.9-1 mm thick. These were incubated for 48 hr at 37°C in a high-humidity CO₂ incubator to allow the cells to adhere. (The cells of nonattaching lines and imprints of fresh specimens were simply allowed to dry on the slides). The slides were rinsed three times with phosphate-buffered saline (PBS), fixed in acetone for 10 min, and stored at -70°C. These preparations were used for testing human sera by IF. For details of the method see reference 25. The fluorescein-conjugated horse anti-human immunoglobulin serum (Progressive Laboratories, Baltimore, Md.) used in the survey of human sera was shown by immunoelectrophoresis to react with the three major classes of human immunoglobulins: γM, γG, and γA. Class-specific fluoresceinated antisera to human immunoglobulin were purchased from Hyland Laboratories, Los Angeles, Calif.

**Assay of Filtrates for Focus-Forming and Antigen-Inducing Capacity.** The two indicator cell lines were WI-38 and SF human fibroblasts (see above). Filtrates were prepared by centrifugation of medium from 3 to 4-day old cultures for 15 min at 1000 g, and by passage of the supernate through a 0.22 μ Sweeney Millipore filter (Millipore Filter Corp., Bedford, Mass.) (with simultaneous controls to exclude passage of cells or bacteria). The indicator cultures were prepared by seeding 1.5 X 10⁵ cells/ml in MEM with 15% FCS in T-30 flasks. A day later the growth medium was replaced by 2.5 ml of filtrate with 2.5 ml of MEM with 5% FCS. The cultures were then kept for 2 hr at room temperature, followed by 48 hr at 37°C in a humidified CO₂ incubator. The medium was changed thereafter twice weekly. The cultures were examined periodically for foci and for IF + cells (see above).

**Electron Microscopy.** The following four fixation procedures were used selectively for individual samples. (a) Fresh tissues were treated with Karnovsky's fixative for 2 hr at room temperature, and after rinsing with Ringer's solution were fixed in cold 1% osmium tetroxide for 1 hr. (b) Cell pellets and Millipore filters were prepared by fixation in cold 2% glutaraldehyde for 20 min, washed three times with Millonig's buffer solution, and exposed to cold 1% osmium tetroxide for 1 hr. (c) Adherent cultures were fixed in situ with cold 2% glutaraldehyde for 20 min. Cells comprising foci were scraped off with a rubber policeman, centrifuged in Millonig's buffer solution, and fixed in cold 1% osmium tetroxide for 1 hr or in Dalton's chrome-osmium for 30 min. After fixation, samples were kept overnight in cold 0.5% uranyl acetate, then dehydrated in alcohol, and embedded in Epon. (d) Adherent cultures on plastic surfaces were washed with PBS, fixed in 2% glutaraldehyde for 1 hr, washed with PBS again, and then postfixed in Dalton's chrome-osmium for 30 min. After dehydration with alcohol, the cell sheets were exposed to progressively increased concentrations of resin in alcohol, and then embedded in Epon. Regions to be examined further were selected by light microscopy, cut out with a cork borer, and fastened with epoxy glue to a prepolymerized Epon block.

Thin sections were cut on a Sorvall MT-2 ultramicrotome (Fram Sorvall, Inc., Norwalk, Conn.) with a diamond knife, stained with 0.5% uranyl acetate in alcoholic solution and lead citrate according to Reynolds (26), and examined with a Siemens Elmiskop IA electron microscope.

**Optical Shadow Casting.** Method of Hlinka and Sanders (27).

**Tests for Mycoplasma (PPLO).** These tests were carried out independently by Dr. D. Armstrong (28) and by Dr. J. Fogh (29) of the Sloan-Kettering Institute, New York and by Dr. L. Hayflick of Stanford University, Palo Alto, Calif., to whom we express our thanks.
Notes on the Morphology and Growth of the Sarcoma Lines in Culture.—Primary cultures (MEM:15% FCS) of the 11 osteosarcomas, 1 leiomyosarcoma, and 1 fibrosarcoma that we have examined consisted initially of a mixed population of cells, areas of epithelial cells alternating with sheaves of fibroblast-like cells. In subsequent passages the fibroblastic cells predominated, showing both fascicular and random orientations in the same culture. The morphology of these fibroblastoid lines ranged from grossly irregular patterns of growth to patterns not clearly distinguishable from cultures of normal fibroblasts. However, after fibroblastoid growth had become established (Fig. 1 a), this could be altered to a more epithelioid type (Fig. 1 b) by changing to a different culture medium or by changing the serum supplement from FCS to human serum. After culture for several months, two of the osteosarcoma lines (OS1 and OS2) exhibited increasing numbers of a cell of distinctive morphology best seen in cultures which had not yet reached confluence. The main feature of this cell is its several long, radiating cytoplasmic processes (Fig. 1 c).

The three liposarcoma lines consisted of fusiform cells and of epithelioid cells containing many fat droplets (Fig. 1 d) which disappeared on passage. The chondrosarcoma and neurosarcoma lines resembled the osteosarcoma lines in being composed mainly of epithelioid and fibroblastic cells, but they also contained some giant cells which were lost in later passage.

Usually the lines started to proliferate and could be subcultured only after some 4–6 wk in culture. After that, the usual passage interval was monthly for 2 or 3 months, and then the passage interval was stable at about 2 wk.

Foci of Altered Cells in Sarcoma Lines in Culture.—Three of the sarcoma lines, identified in Fig 2, developed colonies of altered cells after 4–5 months in culture. These consisted of foci of randomly oriented crisscrossing cells piling up on one another (Fig. 3 a). Around the foot of these foci there occasionally appeared darkly granular cells like those shown in Fig. 3 b and c.

The osteosarcoma line studied in greatest detail was OS3B (see Fig. 2). Foci appeared in this line after 4 months in culture. They remained visible for about 1 month, during which two passages were made. After that they disappeared for 8 wk, reappearing monthly for 8–10 days during the next 3 months. They have not been seen again during the subsequent 3 months of observation. The periods during which foci were evident could usually be extended, either by shortening the passage time or by using horse serum or human cord serum as a serum supplement instead of FCS on successive passages. The morphology of the foci also was influenced by the source of the serum supplement. (a) In fetal calf serum the foci began as discrete areas of crisscrossing cells piling up into mounds (Fig. 4 a). The morphology of the inner cells was obscured by the dense granularity at the center of the focus (see below). The foci were fully mature 7–10 days after their appearance. They
Fig. 1. Morphology of cultured human sarcomas. (a) Osteosarcoma OS3B cultured in MEM: FCS (2nd passage). The dominant cell type is fibroblastic. Phase contrast, × 160. (b) Osteosarcoma OS3B grown in MEM: human serum. In human serum the cells are more epithelioid (cf. preceding figure). Phase contrast, × 160. (c) Osteosarcoma OS1 after 6 months in culture in MEM: FCS (10th passage). In the upper part of the field is a characteristic cell with many spidery processes (see Text). Phase contrast, × 120. (d) Liposarcoma in MEM: FCS (2nd passage). The cells are epithelioid, with many cytoplasmic fat droplets. Phase contrast, × 400.
Fig. 2. Summary of results observed with seven cultured human sarcomas. WI, SF = cultures of WI-38 human fibroblasts or human embryonic skin fibroblasts.
FIGs. 3, 4, and 5. Foci appearing spontaneously in cultured osteosarcoma OS3B.

FIG. 3. (a) Sixth passage in MEM: FCS, showing four foci. Giemsa. X 40. (b) Periphery of a focus, showing pigmented epithelioid cells. Phase contrast, X 256. (c) Same as (b). Shadow casting, X 256.
Fig. 4. (a) Conical-type focus formed in FCS, (12th passage). Shadow casting, × 40. (b) Broader plateau-like foci formed in horse serum, (12th passage). Shadow casting, × 40. (c) Higher magnification of focus formed in horse serum, Shadow casting, × 160. (d) Same as (c). Phase contrast, × 160. (e) Morphology of cells at periphery of focus formed in horse serum. Phase contrast, × 400.
remained unchanged for 2-5 days, after which they either decreased in size and finally disappeared, or became detached from the surface, leaving behind a crater rimmed by the remaining cells (Fig. 5). (b) In horse serum the foci were plateau shaped and larger (Fig. 4 b, c, d, and e). These relatively massive foci have been seen to fall away from the glass, and they have been recovered in the supernate. One attempt to propagate them on layers of irradiated cells was not successful. (c) In human cord serum the cells were more epithelioid and details of the foci were less distinct, probably because there were fewer superimposed layers.

It seems that the appearance of foci at a given time in a culture's history is characteristic of that particular line. Thus when the osteosarcoma line OS1 was established in culture a second time, from cells in frozen storage, the foci appeared after approximately the same length of time that preceded their appearance in the original isolate (Fig. 2). A third line was established from frozen OS1 cells and again showed the same time-dependent induction of foci. The same phenomenon has been seen repeatedly with OS3B.

Similar loci have been seen in two other osteosarcoma lines in addition to those shown in Fig. 2.

Repeated tests for PPLO (see Materials and Methods) were performed on cultures showing foci, and were invariably negative.

**Induction of Foci in Cultured Human Fibroblasts by Filtered Medium of Sarcoma Cultures.**—Cultures of WI-38 or SF fibroblasts seeded 24 hr previously in T-30 flasks (see Methods) were exposed to filtered medium from 3 to 4-day old sarcoma cultures. In 12 out of 23 tests foci appeared 1-2 wk later. As a rule 8-15 foci appeared in each culture. In all experiments recorded in Fig. 2 no foci were seen in control cultures not exposed to filtrates.

Filtered cultured medium from other established lines (two mammary adenocarcinomas [AlAb, BT 20], two melanomas [RPMI 4445, RPMI 5966], and one line of Burkitt's lymphoma [P3HR-1]) failed to induce foci. The gross and microscopic morphology of the foci induced by filtrates was similar to that of the foci occurring spontaneously in the sarcoma cultures (Fig. 6). Like the foci that appeared spontaneously, the induced foci persisted in secondary cultures but disappeared in later passages. Several attempts were made to induce foci with filtered medium from focus-positive indicator cultures, but without success.

With the object of raising the titer of the focus-forming agent, sarcoma cells were cocultivated for 12 days with cells from a mammary adenocarcinoma (Lev-III) which contains particles resembling oncornavirus (30, 31). The result was negative; filtrates of the medium showed no increase in focus-inducing activity in comparison with filtrates made directly from the sarcoma culture.

Positive filtrates, inducing foci in indicator cells, could be obtained from lines which themselves had never shown foci and from focus-forming lines
Fig. 5. Stages in the spontaneous evolution of foci in osteosarcoma OS3B cultured in MEM: FCS, (10th passage). X 160.
Fig. 6. Induction of foci in WI-38 human fibroblasts by filtered medium from cultured osteosarcoma OS3B. (a) Control untreated WI-38 cells and (b) WI-38 cells exposed to filtrate of OS3B culture medium 12 days previously, showing a mature focus. Shadow casting, X 40.
which were not showing foci at the time the filtrates were prepared, as well as
from lines which currently exhibited foci. In fact there seemed to be no rela-
tion between the presence of foci in an established line and the ability of fil-
tered medium from that line to induce focus formation (see Fig. 2).

Some tentative statements can be made regarding the physical properties of
the focus-forming agent: it (a) passed a 100 mμ, but not a 50 mμ Millipore
filter, (b) sedimented after 2 hr at 50,000 g, and (c) was inactivated after 1 hr
at 56°C. Focus-forming activity was not demonstrable in filtrates diluted
beyond 1:100.

Electron Microscopy.—

(I) Search for virus: The main purpose of electron microscopy in this study
was to search for the presence of a virus associated with human sarcoma cells.
In no sample, whether from fresh surgical specimen, tissue culture line, focus,
or culture fluid was there convincing morphological evidence for this. The
following list indicates the extent of this negative material. (a) Surgical speci-
mens: osteosarcomas (3), liposarcoma (1), neurofibrosarcoma (1); (b) Primary
cultures: osteosarcomas (4, designated OS1-OS4), chondrosarcoma (1), lipo-
sarcomas (2); (c) Secondary cultures: osteosarcomas (17 samples from 7
different osteosarcomas after 1–10 passages in culture), chondrosarcoma (1,
third passage), liposarcoma (first and second passage), neurofibrosarcoma (1,
first and third passage); (d) Cell-free preparations: pellets prepared by cen trif-
ugation of culture fluids (60,000 g) from focus-positive and focus-negative
sarcoma lines. Millipore filters of porosities of 50, 100, and 200 mμ through
which culture fluids known to have focus-forming activity had been passed.

(II) Ultrastructure of loci: The salient ultrastructural features of the sponta-
neous and induced foci did not differ. There was much intercellular debris,
indicative of cytolysis, accompanied by phagocytosis and by cells in various
states of degeneration (Figs. 7–9). There were often frequent myelin figures
in lysosome-like bodies, and many lipid or secretory droplets, most likely
also indicative of cellular degeneration.

The viable cells in the foci contained few mitochondria, in contrast to the
large numbers characteristic of fibroblasts. The rough endoplasmic reticulum
was often well developed, forming many concentric layers in some cells (Fig. 8).

For comparison we examined foci produced in chick embryo cells by the
Bryan strain of Rous sarcoma virus (cultures kindly provided by Dr. H.
Hanafusa). These showed a striking similarity to the human sarcoma foci
described above. In fact our description of the human cell foci is equally
applicable to the Rous sarcoma virus foci, except that the latter contain
abundant virus.

Immunofluorescence.—

The sarcoma lines: Sera from patients with osteosarcoma were tested by in-
direct IF for reaction with seven sarcoma lines (Fig. 2). Positive reactions (IF+)
Fig. 7. Section of spontaneous focus in culture of osteosarcoma OS3B showing phagocytosis and cells with vacuoles containing lipid or secretory droplets. There is much intercellular debris, which evidently contributes to the bulk of the focus. × 4300.
Fig. 8. Spontaneous focus in osteosarcoma OS3B culture, showing concentric formation of rough endoplasmic reticulum, and remnants of a phagocytosed cell. × 6900.
Fig. 9. Cells of focus induced in embryonic human skin fibroblasts by filtered medium of osteosarcoma OS3B culture. The appearance is similar to that of the spontaneous focus (dissociating cells, myelin figures, phagocytosis, and lipid secretory droplets). As with the spontaneous foci, no virus is seen. × 8000.
were observed on six of them. The IF+ reaction consisted of punctate cytoplasmic fluorescence occurring in 0.01–5% of the cells (Fig. 10). Serial testing at intervals of 2 wk showed the percentage of positive cells to vary from one extreme to the other, even in the same line. In fact a line might become IF– for one or two passages and then become strongly IF+ again. The 7th line, OS3B, remained IF– throughout its entire history of 9 months of culture.

Figs. 10 and 11. Indirect immunofluorescence tests on acetone-fixed cells with serum (1/10) from a patient with osteosarcoma.
Fig. 10. Typical cytoplasmic fluorescence in cultured osteosarcoma OS2 cells. × 400.

*Sarcoma specimens before culture:* IF tests were carried out on imprints of 10 osteosarcomas, 1 liposarcoma, 1 fibrosarcoma, and 1 neurosarcoma before culture. The only positive reactions observed were shown to be due to fluorescent antiglobulin bound to globulin present on or in the cells at the time of their removal from the patient. This reaction may be attributed to the presence of immunoglobulin-forming cells, or to the coating of sarcoma cells with
Fig. 11. WI-38 cells previously exposed to filtered medium from (a) human mammary adenocarcinoma AIAb (control), showing no IF+ cells, and (b) human osteosarcoma OS20 showing typical punctate cytoplasmic fluorescence in a small proportion of the cells. X 400.
immunoglobulin from the patient's serum in vivo. The latter may represent antibody specific for the sarcoma cells, but to demonstrate this would require analysis of the eluted globulin, which has not so far been attempted.

In eight additional cases, sarcoma cells were studied both at the time the surgical specimen was received, and also serially after culture. All eight cultures were initially IF−, six of them becoming IF+ after 3–4 wk in culture.

**Serological specificity of the IF+ reaction:** IF+ sera from cases of osteosarcoma were tested against the 29 nonsarcoma lines listed in Materials and Methods. These include epithelial and fibroblastic lines, both normal and neoplastic in origin, and 20 cell lines that grow without attaching to the culture vessel. Without exception, these tests were negative.

**Induction of antigen by filtrates:** Filtrates from fluids of 3-day old sarcoma cultures induced the appearance of 0.01–1% IF+ cells in WI-38 monolayers (Figs. 2, 11 a and 11 b). The appearance of the IF+ reaction was similar to that given by the sarcoma cultures themselves. Positive filtrates were obtained from IF− as well as from IF+ sarcoma cultures. The IF+ reaction induced in WI-38 cells by filtrates, like the IF+ reaction of the sarcoma lines themselves, was lost on serial passage. In the relatively few tests conducted so far, the IF+ reaction induced in WI-38 cells was not transmissible to fresh WI-38 cultures.

**Relation of the IF+ reaction to the presence of foci:** It is apparent from Fig. 2 that the IF+ reaction does not depend on the presence of foci in the sarcoma lines. Similarly, the IF+ reaction and the formation of foci may be induced independently of one another by filtrates. This lack of association between the two is illustrated by OS3B (Fig. 2), which consistently exhibited foci and yielded filtrates which induced foci in WI-38 and embryonic skin fibroblasts, but was consistently IF−.

**Survey of human sera for the presence of antibody reactive with IF+-cultured sarcoma cells:** The considerable fluctuation in the expression of antigen by IF+ sarcoma lines posed a problem in obtaining a sufficient number of standard test cells. To overcome this, large numbers of cells were grown and tested for antigen. Frequently these were IF− or had such low activity that they had to be discarded. On those occasions when IF+ cells were sufficiently numerous, the entire yield of cells was used for preparing slides (see Materials and Methods). One such batch of slides was used for the survey shown in Table I. Smaller surveys from other batches of slides have given generally similar results.

Clearly the occurrence of antibody is not limited to patients with sarcoma, nor do these patients have significantly higher titers of antibody. The presence of antibody is equally common in normal subjects, including members of the families of osteosarcoma patients, and in patients with other types of cancer, with two possible exceptions: First, fewer positive reactions, and generally lower titers, were observed in patients with chronic lymphatic leukemia or
Hodgkin's disease; this may be related to immunosuppression in these groups of patients, arising from the disease itself or from therapy. Secondly, four of six patients with neuroblastoma showed particularly high titers, suggesting the need for further evaluation of this group of patients in regard to the occurrence of the sarcoma antibody, and for studying neuroblastoma cultures for the presence of the sarcoma antigen.

TABLE I
Survey of 216 Human Sera for the Presence of Antibody Reactive with IF*-Cultured (OS1) Sarcoma Cells

| Serum donors diagnosis                      | Titer       | Number of cases |
|---------------------------------------------|-------------|-----------------|
|                                             | <1/10 | 1/10 | 1/40 | 1/160 |
| Osteosarcoma                                | 3     | 5    | 12    | 6    |
| Family members of osteosarcoma patients     | 1     | 2    | 13    | 3    |
| Chondrosarcoma                              |       |      | 5     |      |
| Liposarcoma                                 | 2     | 0    | 3     | 0    |
| Chronic lymphocytic leukemia                | 6     | 3    | 1     | 0    |
| Chronic myelocytic leukemia                 | 5     | 2    | 0     | 0    |
| Hodgkin's disease                           | 4     | 5    | 1     | 0    |
| Acute lymphoblastic leukemia                | 2     | 5    | 3     | 0    |
| Acute myeloblastic leukemia                 | 2     | 5    | 2     | 1    |
| Lymphosarcoma                               | 1     | 5    | 4     | 0    |
| Reticulum cell sarcoma                      | 2     | 4    | 4     | 0    |
| Ca. colon                                   | 1     | 3    | 6     | 0    |
| Ca. breast                                  | 1     | 1    | 6     | 1    |
| Ca. skin                                    | 2     | 1    | 5     | 0    |
| Ca. lung                                    | 2     | 2    | 6     | 0    |
| Ca. postnasal space                         | 2     | 0    | 7     | 1    |
| Melanoma                                    | 0     | 2    | 6     | 2    |
| Neuroblastoma                               | 1     | 0    | 1     | 4    |
| Normal subjects                             | 3     | 2    | 23    | 3    |

There is no clear indication of an increase in the frequency of sarcoma antibody, or of increased titer, associated with any of the diseases listed, including sarcoma itself, in comparison with a normal population.

Serial tests for changes in serum antibody titer: Serial tests of serum over a period of several months have been carried out on 10 normal subjects and on 6 patients with sarcoma or some other form of cancer. In general, titers have not shown significant fluctuation with time, with the following exceptions: (a) One of the normals (GG) showed a rise in titer from 1/10 to 1/160 over a 5 month period during which he was working with sarcoma cultures. (b) in 5/6 cases, surgical removal of osteosarcoma was followed by a 4-fold or greater rise in titer; surgical removal was not followed by a rise in titer in 5/4 cases of malignancy other than sarcoma. (c) In one case, recurrence of osteosarcoma after surgery was accompanied by a marked fall in antibody titer.
Other serological data: According to tests with class-specific immunofluorescent antiglobulins, IF activity is due predominantly to \( \gamma M \) antibody, and to a lesser extent to \( \gamma G \). Standard IF\(^+\) sarcoma cultures did not react with reference antisera to the group antigens of murine leukemia virus or feline leukemia virus. Antisera to mycoplasma orale 1 and 2 and laidlawii were also negative (except with cultures deliberately infected with these organisms). IF antibody was not detected in human cord serum, or in fetal or adult bovine serum.

DISCUSSION

The two observations that most require discussion are the foci and the shared antigen in the sarcoma lines. Both may be induced, albeit irregularly, in cultures of human fibroblasts. At face value, these findings suggest a human sarcoma virus homologous with the sarcoma viruses of chicken, mouse, and cat. If characteristic oncornavirus had been demonstrable by electron microscopy, this view would have been strengthened. In fact, this was not the case, despite extensive search. On the other hand, the lack of such particles does not weigh heavily against an etiological role for oncornavirus, not only because of the technical problem of detecting small numbers of virions by electron microscopy, but also because there are many examples of nonproducing virus-induced tumors in animals. So interpretations at this point must rest on the significance that can be attached to the foci and to the serological reaction.

The Foci.—Although it is clear that the foci are made up of cells with altered morphology and behavior, are they comparable with the foci of transformed cells induced by animal sarcoma viruses in vitro? These themselves do not conform to a constant pattern, so there is no absolute criterion here, nor are there any specific changes recognizable by electron microscopy which serve to distinguish a transformed cell from a normal cell. The Rous foci which we compared with the human foci (see Results) showed several points of similarity, although C-type virus was demonstrable only in the former. Technical artifacts mimicking focus formation (such as inadequate trypsinization leading to the formation of cellular aggregates) are unlikely, for the following reasons: (a) The foci do not appear immediately after passage but in developing cultures which previously showed no sign of them. (b) There is a periodicity to the presence of foci in culture, so that in certain cases their appearance can be predicted, e.g. in osteosarcoma line OS1 (Fig. 2) which on three separate occasions exhibited foci at the same period after establishment of the culture, either from the primary isolate or from cells stored in the tumor bank. (c) The dark round cells (see Fig. 3b) which appear to migrate from the human sarcoma foci are similar to those seen in foci transformed by Rous sarcoma virus. (d) The foci can be induced by filtrates.

A number of factors make it difficult to study the foci and the agent which produces them. Although the foci appear with a certain regularity in some
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sarcoma lines, and although their duration can be prolonged to some extent by manipulation of the tissue culture constituents, nevertheless they ultimately disappear and one is forced to start afresh. Again, their fate is a mystery. We have not been able to establish whether all the cells die, or whether the detachment of foci we have sometimes observed, leaving behind a crater, is their usual end. On one occasion we were able to place such a detached focus on a feeder layer of irradiated cells, and the cells of the focus died. We hope to learn more about the focus-forming cells by cloning them, if this proves to be possible.

Study of the focus-forming agent itself is complicated by the unexplained irregularity of its transmission to normal fibroblasts by filtrates. This may be attributable to its evident low titer, which could also explain why serial transmission has not been successful. We are aware of the possibility that the genotype of the indicator cell may control susceptibility to the agent, as it does in the case of Rous sarcoma virus (32), and for this reason we are testing as many human fibroblast cultures as possible in the hope of finding one of high susceptibility. On the same line of argument, the fibroblast is possibly not the indicator cell of choice for a virus suspected of tropism for osteoblastic cells. Finally, foci of similar type have been observed, rarely, in fibroblast cultures maintained in routine passage. This occurred on two occasions in our human skin fibroblast line, and on one occasion in a recently acquired WI-38 fibroblast line. These three observations were made during the study of the sarcoma lines, and so extrinsic contamination cannot be ruled out as an alternative to an intrinsic focus-forming agent with rare expression (see below). Whatever the explanation, it must be emphasized that no such spontaneous foci were observed in any of the control cultures included in the transmission studies summarized in Fig. 2.

The Sarcoma Antigen.—Assessment of the serological reactions seems somewhat more straightforward, although variability in the expression of antigen in the sarcoma cultures, and variability in the induction of antigen in fibroblast indicator cells, are similarly troublesome. We are coming to regard quantitative variation in antigen expression as perhaps typical of cellular antigens specified by viruses, for it has been seen with antigens specified by Gross virus in the mouse (unpublished personal observation) and in man by the Epstein-Barr virus associated with Burkitt’s lymphoma (33, 34). Another point of relevance to viral etiology, and perhaps connected with the variable expression of antigen, is that in the sarcoma system, as in the Burkitt system, only a proportion of the cells give a positive reaction at a given time. Qualitatively, expression of the antigen has proved so far to be limited to the IF+ cells.

In recent experiments, the proportion of positive cells in the sarcoma lines could be raised by growing the cells on the glass slides for 7 days rather than for 48 hr only (see Methods) before their use in IF tests.
sarcoma lines and to fibroblasts exposed to filtrates of these lines. In contrast to this disease-related pattern in the occurrence of antigen, the occurrence of antibody shows no apparent relation to disease, in fact the majority of subjects with or without malignant disease have antibody. The agent inducing this antigen therefore must be very widespread. Pursuing the analogy with Burkitt's lymphoma, which has been studied more intimately and where it is known that virus and virus-related antigen can be uncovered by culturing the leucocytes of healthy subjects, we may anticipate that a more extensive survey of human normal and malignant tissues will reveal antigen in some tissues other than sarcomas themselves, if not in human fibroblasts then in normal osteoblastic cells for example. Another prediction from analogy with Burkitt's lymphoma (35, 36) is that there should be little or no antigen expressed in primary tumors, and that antigen should appear in detectible quantities after a period of culture in vitro (possibly because the cells have been separated from an immune environment). This is indeed so, for all sarcoma specimens we have tested before culture have been negative.

Our results differ from those of Morton and his colleagues (10, 14) in two respects. In their hands, (a) the original surgical specimens gave IF+ reactions, and (b) antibody was found in greater frequency and higher titer in patients and their relatives than in the population at large. In both their study and our own, however, the reaction was transmissible to normal human fibroblasts by sarcoma culture filtrates. These differences in serological findings may originate from differences in the spectrum of antigens demonstrable under different test conditions, for probably it is a complex of antigens we are dealing with, as in the case of Burkitt's lymphoma antigens, rather than a single determinant.

*Association between Focus Formation and the Appearance of Antigen.*—In general these two characters were expressed independently of one another, whether spontaneously in the sarcoma line or by induction in fibroblasts exposed in sarcoma culture filtrates. Thus the line OS3B (Fig. 2) which exhibited foci spontaneously, and also repeatedly induced foci in fibroblast cultures, was consistently negative for IF antigen. On the other hand, the OS1 line repeatedly showed concurrent appearance of the foci and antigen after approximately the same period of cultivation. Their apparent dissociation in other instances is perhaps a consequence of the undoubtedly threshold levels of sensitivity at which we are working in detecting either phenomenon.

*Comment.*—This report is intended to be primarily descriptive and to illustrate the application of a group of techniques to the study of a type of human cancer. The evidence presented is far less than is required to sustain a viral theory for the origin of human sarcomas, but it is more than sufficient to indicate further investigation in that direction.
SUMMARY

In a study of human sarcomas maintained in culture for periods up to two years, the following observations were made.

The most prominent cell type in serially cultured osteosarcomas was fibroblastic in appearance. After 16–20 wk in culture some lines spontaneously developed foci of altered cells resembling the foci produced in monolayer cultures by oncogenic viruses. The presence of these foci in the sarcoma cultures was transient, and usually they did not reappear; but in one instance they recurred with a characteristic periodicity of several weeks. From one of the sarcoma lines, in which foci appeared after 5 months in culture, two subcultures were established from stored frozen cells and these both exhibited foci after approximately the same lapse of time. The same phenomenon has been seen with another line, suggesting that the time of appearance of foci is characteristic for particular sarcomas. Foci of similar type could sometimes be induced in monolayer cultures of human fibroblasts by filtered medium from cultured sarcomas; this bore no relation to the presence or absence of foci in the sarcoma cultures at the time the filtrate was prepared. Electron microscopy of the spontaneous and induced foci, and of the sarcoma cultures, revealed no demonstrable virus.

12 out of 15 sarcoma cultures contained an antigen (S) demonstrable by indirect immunofluorescence with human sera. It was not present in any of the original sarcoma specimens, nor in any culture lines other than sarcomas. At least 3–4 wk in culture appear to be required for its demonstration. The antigen was cytoplasmic, occurred in only a small proportion of the cells, and was unpredictably variable in its expression, even in the same culture line. It could be induced in monolayer cultures of human fibroblasts by filtrates of medium from sarcoma cultures. As with the foci, the induction of S antigen in indicator cultures was not dependent upon the expression of antigen in the sarcoma line from which the filtrates were obtained. There was no association between the presence of foci and of antigen, nor was there any apparent relation between the ability of filtrates to induce foci and their ability to induce antigen. 80% or more of the general population have S antibody, and the titer of antibody in patients with sarcoma is no higher than in normal subjects. Thus, as in the case of Burkitt’s lymphoma antigen, it appears that most individuals have been exposed to S antigen. But unlike Burkitt’s lymphoma, no relation has so far been established between any particular disease and a corresponding high titer or frequency of occurrence of S antibody.

The occurrence of foci of altered cells and of a common antigen, and the transmission of these two characters to indicator cells by filtrates, are all suggestive of a virus specifically associated with human sarcomas, one to which the general population is widely exposed, as indicated by the prevalence of antibody.
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