Studies of the Antigens of Human Tumors.

I. Demonstration of a Soluble Specific Antigen in HeLa Cells and Some Human Tumors*

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SUMMARY

The presence of a soluble specific antigen serologically identical with an antigen found in HeLa cells has been shown in 31 per cent of human malignancies. Similar material has not been isolable from nonmalignant adult or fetal human tissues. According to the accumulated statistical data, there seems to be no correlation between the degree of malignancy of the diseased tissue and the presence or absence of the antigen.

The existence of immune mechanisms in the biology of human cancer has been looked at from two rather separate, but often overlapping, points of view. One of these has dealt with the phenomena of transplantation immunity and associated reactions mediated by cells. This work has been reviewed recently by Brent (2) and Southam (11).

The other avenue of approach has been concerned with those immune reactions involving circulating antibody. Investigations along these lines have been directed toward the detection of antigens unique to malignant tissue and the detection of circulating antibodies thereto.

During the past several years reports have come from a number of workers describing the presence of unique antigens in malignant tissues which were not demonstrable in a variety of nonmalignant tissues. Among these have been the reports of Björklund et al. (1), Zilber (13, 14), Taylor et al. (12), de Carvalho (5), McKenna et al. (9, 10), and Charney and Coriell (3). With the exception of the work of Björklund (1), the other workers have described soluble antigens demonstrable by complement fixation (9, 10, 12), anaphylaxis (13), and gel diffusion (3, 5, 14). Our studies have been extended to include serologic comparisons of 123 human malignancies, twenty normal adult tissues, seven embryonic tissues, and ten benign tumors.

MATERIALS AND METHODS

Preparation of antigens—HeLa cells either were purchased as frozen pellets from Microbiological Associates, Inc., Baltimore Biological Laboratories, or were grown in our laboratory in Eagle's Spinner Medium supplemented with 10 per cent normal calf serum. Human tissues were received either as fresh surgical specimens from the Department of Surgery of the Hospital of the University of Pennsylvania and the Graduate Hospital of the University of Pennsylvania, or from the Departments of Pathology following necropsy. All HeLa cells and tissues were stored at —20°C. until extracted. HeLa cells and tissues were treated with fluorocarbon as described previously (10). Tissues and cell extracts were stored at —20°C. until assayed. Briefly, the extraction procedure consisted of homogenizing the tissues at high speed for 5 minutes at 4°C. in distilled water to yield 20 per cent suspensions. After centrifugation at 3000 × g for 10 minutes, the pellets were discarded. These aqueous extracts were called crude antigens (C). One-half volume of Genetron 113 (Allied Chemical Corporation) was added to the crude supernatant fluids and homogenized for 1 minute as above. Following centrifugation the supernatant fluids were treated with fluorocarbon as before for a total of 5 times. The final aqueous supernatant fluids were called Genetron antigens (G).

Preparation of antisera.—Antisera to extracts of tumors and HeLa cells were produced in albino rabbits, fed and watered ad libitum, by the weekly intramuscular injection of C or G antigens emulsified with an equal volume of complete Freund's adjuvant. Each rabbit received six injections of 1 ml. of the antigen-adjuvant mixture containing 1 mg. of tissue protein per ml. as determined by the method of Lowry et al. (8). Animals were bled by cardiac puncture 10-12 days after the last injection. All sera were stored at —20°C. in small portions until used and then kept not more than 2 weeks at 4°C. γ-Globulin was separated from HeLa (G) antiserum as outlined in Kabat and Mayer (6). The separated γ-globulin was lyophilized and used as either a 5 or 1 per cent aqueous solution.

Serologic assay system.—The assay system used was a micro-double gel diffusion test with 0.8 per cent Ion Agar #2 in veronal buffered saline at pH 7.4. The technic was...
RESULTS

Demonstration of HeLa specific antigen.—A typical configuration of nonidentity between two antigen-antibody systems is shown in Figure 1. Here the MT-1 (C) antigen-antibody system is shown between wells 1 and 3, whereas the HeLa (G) antigen-antibody system is between wells 2 and 4. Figure 2 shows the persistence of two precipitin bands between HeLa (G) antigen and its antibody after absorption of both antisera with an equal volume of MT-1. That an equal volume of MT-1 was sufficient for complete absorption of any common antibodies from the antisera is demonstrated by the complete absence of precipitin lines between well 3 (MT-1) and well 1 (MT-1 Ab and MT-1).

When the geometry of the hole patterns in the agar was changed and the immune globulins from the anti-HeLa serum used as a 5 per cent solution, it became more apparent that the HeLa preparation was a mixture of antigens (Fig. 3). The identity between HeLa G Ag and MT-2 C Ag should especially be noted.

A series of absorptions of the HeLa (G) antiserum is shown in Figures 4–6. In Figures 4 and 5 the peripheral wells contain the antiserum with MT-1 at 10 mg/ml of protein in from 1 to 64 volumes to 1 volume of antiserum. It is readily evident that more than 16 volumes of MT-1 were required to completely remove all precipitin bands. After being de-stained in two or three changes of 2 per cent acetic acid, the slides were counter-stained for 3 minutes in 0.1 per cent acridine orange. Excess acridine orange was removed in 2 per cent acetic acid. The agar was dried for 2–4 hours at 37°C. and the film preserved with a coverslip.

Absorption of antisera.—Twenty per cent aqueous homogenates were made of three separate samples of human liver and spleen in equal parts. These tissues were obtained at autopsy from a patient with Hodgkin’s disease (MT-1), a patient with cardiovascular disease (NT-1), and a patient with carcinoma of the lung (MT-2). After centrifugation at 31,000 X g for 1 hour the supernatant fluid was precipitated by 65 per cent saturation with (NH₄)₂SO₄, the precipitate redissolved in distilled water to contain 10 mg/ml of protein, and the preparation called crude (C) antigen. Antisera to tumor G antigens and HeLa G antigens were mixed with varying amounts of MT-1, NT-1, and MT-2, and the mixtures were placed in the holes for gel diffusion. Unabsorbed sera were diluted with a volume of 0.85 per cent NaCl equal to the volume of absorbing material.

Dilution of the antiserum with saline produced the same configuration of nonidentity between two antigen-antibody systems is shown in Figure 1. Here the MT-1 (C) antigen-antibody system is shown between wells 1 and 3, whereas the HeLa (G) antigen-antibody system is between wells 2 and 4. Figure 2 shows the persistence of two precipitin bands between HeLa (G) antigen and its antibody after absorption of both antisera with an equal volume of MT-1.

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Results, indicating that specific antibody was not absorbed by MT-1 but merely diluted beyond detectability. In Figure 6 the same antiserum was absorbed with from 1 to only 4 volumes of HeLa (G) antigen at 1 mg/ml of protein in wells 1–3. Well 4 contained the antiserum plus 4 volumes of MT-1 for comparison purposes only. Here it may be seen that 2 volumes of HeLa (G) antigen removed all precipitin bands.

Presence of an antigen identical with HeLa (G) antigen in some human tumors.—Human tissues were homogenized in distilled water to yield 20 per cent suspensions. After centrifugation at 3000 X g for 10–15 minutes the supernatant fluids were placed in peripheral wells around a central well containing HeLa (G) antiserum and 2–4 volumes of MT-1 or NT-1. A typical picture is given in Figure 7. A breakdown of the tissues by organ location is shown in Table 1.
Table 2 shows a similar breakdown by diagnosis by cell type of 80 tumors of which the full pathologic report was available. There appears to be no predictable pattern as to the presence or absence of the antigen in malignant tissue with respect to either location or cell type.

The nonmalignant tissues tested for the presence of an antigen identical with HeLa were all negative when assayed against HeLa antiserum absorbed with either MT-1 or NT-1. The adult normal tissues included six livers, four spleens, four kidneys, three skeletal muscle samples from three separate patients, one lung, and two uteri. The embryonic normal tissues included two livers and one each of spleen, kidney, brain, umbilical cord, and placenta. Normal tissues came from twelve individuals. All ten benign tumors studied to date have been uterine leiomyomas.

When the presence of a seemingly specific antigen had been determined in some human tumors it was decided to prepare a large quantity of antiserum to a G antigenic preparation. Early studies revealed that the amount of total protein in the G antigens derived from HeLa cells was relatively constant at 1 mg/ml of final supernatant fluid but that the total protein in the G antigens from human...
Fig. 7.—Typical pattern of identity of human tumors with HeLa G antigen.
Center well: HeLa G Ab. + 2 volumes MT-1 C Ag.
1. HeLa G Ag.
3. Adenocarcinoma of bowel.
4. Melanosarcoma.

Fig. 8-10: Test for specificity of an antigen in a human melanoma.

Fig. 8—Unabsorbed γ-globulin.
Center well: T 164 G 5% Horse γ-globulin.
1. MT-1 C Ag.
2. T 164 G Ag.
3. NT-1 C Ag.
4. MT-2 C Ag.

Fig. 9—γ-Globulin absorbed with normal human tissue extract.
Center well: T-164 G 5% horse γ-globulin + 5 volumes MT-1 C Ag.
1. MT-1 C Ag.
2. T 164 G Ag.
3. NT-1 C Ag.
4. MT-2 C Ag.

Fig. 10.—Identity of T 164 G antigen with HeLa antigen.
Center well: T 164 G 5% horse γ-globulin + 5 volumes MT-1 C Ag.
1. T 164 G Ag.
2. HeLa G Ag.
3. MT-1 C Ag.
4. MT-2 C Ag.
tumors varied from about 70 μg. to 5 mg/ml. A large mass of metastatic melanoma T-164, which contained an antigen identical with the G antigen from HeLa cells, was extracted with fluorocarbon as described above. The total protein of the final supernatant fluid was found to be 4 mg/ml. This was emulsified with an equal volume of complete Freund’s adjuvant, and a 1500-pound mare was immunized by six weekly injections of 5 ml. of the emulsion into the flank muscles. The mare was bled from the jugular vein 2 weeks after the last injection, and the γ-globulin was separated from the serum as described by Kabat and Mayer (6).

The results of testing a 5 per cent aqueous solution of this equine γ-globulin are shown in Figures 8–10. Somewhat in contrast to the HeLa G Ag.-G Ab. system the major precipitin band formed by the T164 G Ag.-G Ab. system appears to be due to a common component of human tissues. (The heavy internal rectangle in Fig. 8.) However, the antibody to this material is absorbable by MT-1 C Ag., leaving behind a precipitin band showing identity with the HeLa G antigen (Fig. 10).

**DISCUSSION**

The results presented in this paper demonstrate the presence of at least one soluble antigen in 31 per cent of human malignancies which is identical with an antigen present in HeLa cells. The same or similar antigenic material was not demonstrable in several nonmalignant adult and embryonic tissues. A second soluble antigen has appeared less consistently. The antibody to this second antigen was not absorbed by MT-1, suggesting specificity to HeLa cells. The band formed by this second antigen may be seen most clearly between wells 2 and 4 in Figures 1 and 2 and as an inverted “U” in Figure 10. Its identity with an established human cell line of malignant origin. The HeLa cell was chosen as perhaps the best choice of the HeLa cell also afforded a relatively stable and potentially inexhaustible supply of antigenic material.

The studies described in this paper were undertaken to determine whether human tumors from a variety of locations in the body possessed any antigenic identity or similarity with an established human cell line of malignant origin. The HeLa cell was chosen as perhaps the best known and most studied of the human cell lines. The choice of the HeLa cell also afforded a relatively stable and potentially inexhaustible supply of antigenic material.

The finding of antigenic material identical with soluble antigenic material in HeLa cells in 31 per cent of human tumors, irrespective of the organ, tissue, or origin, does not preclude the possibility of the existence of other antigens in the tumors which may not be present in nonmalignant tissue. However, intensive immunization of a horse with a tumor G extract did not reveal the presence of tumor-specific soluble antigens other than that showing identity with the HeLa G antigen (Fig. 10).

Of particular interest was the finding that the HeLa-specific antigen was also found in a 20 per cent aqueous homogenate of liver and spleen from a patient with carcinoma of the lung (MT-2). Although the liver contained several metastatic nodules, sections were taken which were at least grossly free of metastases. Of the six other adult livers, none has shown evidence of the HeLa-specific antigen, even though three of the livers came from patients with metastatic disease.

It was believed possible that the degree of malignancy of the tumor might result in the appearance of the antigen in question. When the clinical records of the 80 patients with the most complete data available were reviewed, it was found that the presence or absence of metastatic lesions in the patient had no relation to the appearance of the antigen in the tumor, since one-half of the total number of metastatic tumors showed no evidence of antigen identical with HeLa.

Since there was no obvious pattern regarding the presence or absence of HeLa-specific antigen in human tumors, an explanation on the genetic level might be sought. The presence of the antigen in question may be due to a particular state of ploidy of the cells. This state of ploidy may manifest itself in HeLa cells and in certain other cells which are also malignant. Other cells, equally malignant by clinical and/or pathologic criteria, may not evidence the particular state of ploidy required for the formation of the antigen in question. In this connection, Leuchtenberger et al. (7) showed that normal or diploid human cells contain very similar basic mean amounts of DNA regardless of the tissue of origin. About 30 per cent of malignant tissue contained up to twice as much DNA, whereas 24 per cent contained an amount of DNA suggestive of tetraploidy. In the same paper a correlation was shown between advancing age and the amount of DNA in normal liver. If indeed the increased DNA content were due to heteroploidy and not to malignancy, this might explain our finding of the presence of HeLa-specific antigen in an apparently normal liver from a patient with metastatic adenocarcinoma of the lung (MT-2).

At present little is known concerning the biochemical nature of the material or its physical characteristics. At the present state of purification of the antigen, chemical and physical studies would have little value, since it is evident that none of the preparations was antigenically homogenous. Preliminary experiments have shown the presence of ribonucleo-protein in the antigenic preparations, but it cannot be stated with certainty that the RNP present is indeed the material in question. Relatively little can be said at present regarding the quantitative relationships of the HeLa G antigen and its antibody. When 5 per cent solutions of either rabbit or horse immune globulins were used, no reaction showing identity with the HeLa G antigen was seen when MT-1 or NT-1 at 10 mg/ml of total protein were tested against them (Figs. 3, 8). Conversely, as little as 20 μg. of specific HeLa G antigen could be detected. However, this weight is based on total protein, since the antigens have not been separated as yet, and is, therefore, somewhat high.

Preliminary studies indicate that the antibodies to the HeLa extract described here have some degree of cytotoxicity to HeLa cells. However, the antigenic preparations contained multiple components (Figs. 3, 8), and it has not been determined which of these components may have been responsible for the cytotoxic antibody.

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