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# A Tumor-Associated Antigen in Human Nephroblastomas

**(Wilms' tumor/virus/immunoelectrophoresis/immunofluorescence)**

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**ABSTRACT Antisera prepared against extracts of human nephroblastomas (Wilms' tumors) allowed the characterization in these extracts, and in a few extracts of tumors of other organs, of a tumor-associated antigen. This antigen is different from all previously described antigens, and was named W antigen (W for Wilms). It does not seem to be a carcinoembryonic antigen, and hence it could be of viral origin.**

Immunochemical studies have led to the discovery of several tumor-associated antigens, all of fetal origin. Two of them have been isolated, and their usefulness in diagnosis and surveillance of some carcinomas has been well documented: they are the $\alpha$ fetoprotein (1, 2) and the carcinoembryonic antigen of digestive tumors (CEA) (3, 4). Others, such as the fetal sulfoglycoprotein (5), carcinofetal glial antigen (6), and the $\gamma$ fetoprotein (7) have not been isolated and are less well known. We describe in this paper an antigen that is associated with human nephroblastomas (Wilms' tumors) and is apparently absent from normal adult and fetal tissues. Its origin is thus a matter of conjecture.

A short report of another group (8) described precipitin lines related to tumor-associated antigens in nephroblastomas. Whether these antigens are related to ours is not known.

## MATERIALS AND METHODS

### Materials

*Nephroblastomas.* 33 primary tumors were obtained after surgery. We also used four metastases, two from surgery and two from necropsy.

*Cancers of Different Organs.* Among kidney carcinomas of adult type, we studied three individual samples and a pool of three others. Children's tumors were 13 neuroblastomas, two teratomas, and one dysembryoma. Five pools of mammary carcinomas were studied, each made of several samples as well as pools of carcinomas of the stomach and the colon. In this category are also included 16 cancerous gastric fluids. Individual samples of carcinomas of different organs were used: thyroid, lung, parotid gland, and brain (gliomas). An ovarian cyst fluid was also studied. Leukosic blood samples were studied. One was obtained from a patient with acute lymphoblastic leukemia. It was large and was extracted in the same manner as tissues. The others were only smears: two from acute lymphoblastic leukemia, two from chronic lymphocyte leukemia, one from chronic myeloid leukemia, and one from monoblastic leukemia.

*Normal Organs and Secretions.* Six noncancerous kidneys were studied. Three were removed because of different diseases (tuberculosis, pyelonephritis, and polykystic disease), three were obtained at necropsy from patients who died from different diseases, cancerous or not. A pool of 18 kidneys of children who died from congenital cardiopathies was studied. Other noncancerous organs obtained at necropsy were: livers, adrenals, lung, heart, thymus, and epididymis. Those obtained after surgery were: colonic and gastric mucosae, spleen, and urinary bladder. Different kinds of mucus secretion were: gastric mucus, jejunal juice, and pools of saliva.

*Fetal Organs* were generally obtained from the third to the sixth month of gestation: kidney, liver, lung, adrenals, muscle, epididymis, urinary bladder, skin, stomach, and colon. We once obtained some organs from an 8-cm long embryo (about 2 months of gestation). The kidney was then a mesonephros. We also obtained a pool of fetal sera, placentas, and extracts of umbilical cord.

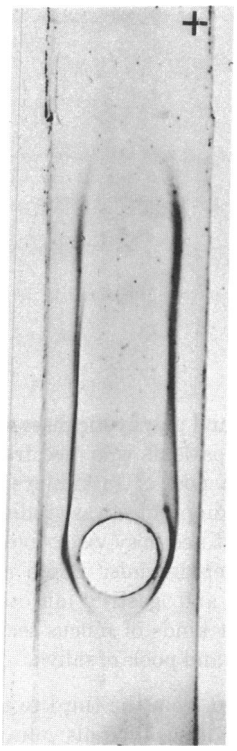
### Methods

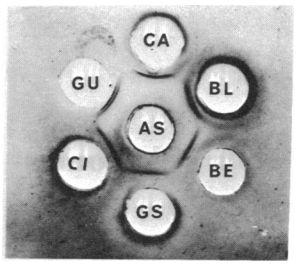
*Extraction of Nephroblastomas* was often difficult because of the large amount of mucus contained in these tumors.

(*a*) *Saline extraction.* The tumor was cut in small pieces and washed with magnetic stirring in phosphate-buffered saline (pH 7.4). It was then suspended in 0.02% EDTA solution in phosphate buffer, pH 6.8, for 20 min as described by Allerton *et al.* (9), homogenized in an Ultraturrax apparatus, and centrifuged for 15 min at 6000 rpm (5500 $\times$ *g*). The precipitate was suspended in phosphate-buffered saline, homogenized again, and centrifuged. The second supernatant thus obtained was often opalescent. The two supernatants, called $E_1$ and $E_2$, were lyophilized. Their protein content was measured by the Biuret method.

(*b*) *Phytic extraction.* Phytic (inositohexaphosphoric) acid extraction was performed on saline extracts or directly on the tissue homogenate, by the method of Courtois *et al.* (10), with slight modifications. To 60 ml of a solution containing 15 mg of protein per ml we added 130 mg of sodium phytate (Merck), followed by concentrated HCl to adjust the pH to 2.2. After centrifugation at 6000 rpm for 20 min, 130 mg of calcium chloride was added to the neutralized supernatant ($CaCl_2$: $2H_2O$, Prolabo, France). This procedure led to almost complete precipitation of phytic acid as calcium salt. After a second centrifugation, the soluble extract was dialyzed against deionized water for 48 hr and then lyophilized.

*Preparation of Antisera.* Rabbits were immunized with saline or phytic extracts of nephroblastomas, according to the following scheme: 5 mg of proteins emulsified in complete Freund's adjuvant injected in the footpad. A second





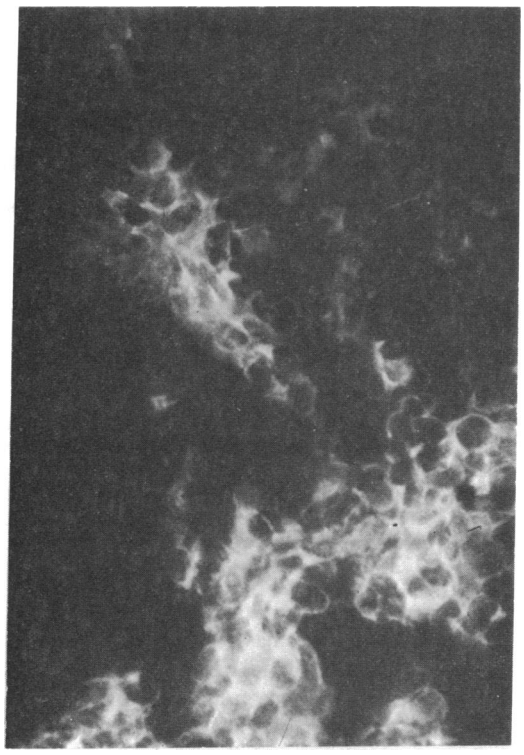



FIG. 1. (*left*). Immunoelectrophoretic analysis of a nephroblastoma extract, reacting with antiserum against W antigen.

FIG. 2. (*above*). Ouchterlony plate showing the precipitin line given by six different nephroblastomas extracts with antiserum (*AS*) against W antiserum.

FIG. 3. (*right*). Immunofluorescence pattern of a nephroblastoma section stained by antiserum against W antigen (×400).

similar injection was generally made 2 weeks later. After a resting period of 2–3 weeks, a series of booster injections was made, each of them consisting of 5 mg of proteins from the alum-precipitated extract. The series generally comprised three injections, the first one being subcutaneous, the others intravenous.

5 Days after the last injection, blood was taken from the ear vein. According to the antibody content of the serum, the animal was exsanguinated or received a new series of booster injections identical to the last one.

Antisera were always absorbed with lyophilized human plasma (40 mg/ml), O and AB erythrocyte stromata (20 mg of protein per ml), and saline extract of normal kidney (50 mg of protein per ml). In many cases, supplementary absorptions were made with extracts of normal, fetal, or cancerous organs, either saline or phytic. The saline extracts were used in concentrations up to 100 mg of protein per ml of antiserum, the phytic extracts being only up to 50 mg of protein per ml of antiserum.

*Immunochemical Methods*, such as double diffusion in gels, immunoelectrophoresis, and counter immunoelectrophoresis, were used. Generally we used 1% agarose gels in 0.05 M borate buffer (pH 8.2). After sufficient diffusion, plates were washed for 5 days in buffered saline, dried, and stained either for proteins by Coomassie Blue or sometimes for polysaccharides by the periodic acid-Schiff method or Alcian Blue.

*Immunofluorescence.* Frozen sections of nephroblastomas and of other organs and smears of leukemic blood were incubated with antiserum (or rabbit normal serum), then with fluorescein-labeled antibody against rabbit globulin antiserum prepared in sheep (Institut Pasteur, Paris). The latter was always diluted to 1/10 with phosphate-buffered saline. All the sections, as well as the smears, were treated with sodium nitrite, as described (11), in order to inhibit a nonspecific fixation of the globulins. Observations were made with an Orthoplan Leitz Microscope, equipped with a Ploem illuminator.

*Enzymic and Biochemical Degradations.* Nephroblastoma extracts were treated by 1% periodic acid, at pH 4, for 4–24 hr or by various enzymes. (*a*) Extracts were incubated with trypsin (Martinet, Paris) for 24 hr. The reaction was stopped with a few drops of 1% diisopropylfluorophosphate (pH 7.4). (*b*) Some were treated with Pronase, either soluble (Calbiochem) 1%, at pH 7 for 4 hr, or insoluble (Enzite Merck). An equal weight of protein and Enzite was mixed for 24 hr at 37°. The reaction was stopped by centrifugation.

Other enzymes used were: neuraminidase (Sigma) 1% (pH 4.5.) for 18 hr at 37°; collagenase (Sigma) 1% (pH 7.4) for 24 hr at 37°; hyaluronidase (Sigma) 1% for 2 and 4 hr at 37°; and ribonuclease and deoxyribonuclease (Sigma) 1%, (pH 7) for 18 hr.

## RESULTS

*Characterization of the Antigen.* Some antinephroblastoma antisera, after absorption with normal plasma, erythrocyte stromata, and normal kidney extract contained antibodies reacting only with nephroblastoma extracts. The precipitin line thus obtained in double diffusion varied greatly in intensity.

In some cases, it was a strong line. It could be observed also in immunoelectrophoresis: it was then a long line, starting from the antigen reservoir and extending to the anode, often through the whole $\beta$ and $\alpha$ zones, sometimes even longer (Fig. 1). This line sometimes split at its fast-moving end, when aged extracts were used.

In other cases, the precipitin line was weaker, due to less antigen. Techniques of increased sensitivity were then necessary to characterize it. It was thus shown that phytic extracts were much richer in antigen than saline ones, and that counter immunoelectrophoresis was more sensitive than the Ouchterlony method. Sometimes weak lines were seen better after protein staining.

On the whole, the vast majority of the Wilms' tumors gave a positive precipitin reaction. Out of 33 tumors studied, 14 were strongly positive both in Ouchterlony and immunoelectrophoretic plates. 11 gave a weak reaction, seen sometimes in saline extracts in Ouchterlony plates, more generally in counter immunoelectrophoresis or with phytic extracts.

Eight tumors were apparently negative. Most of these negative results were obtained when we did not yet use phytic extracts. It is thus possible that these tumors would have been positive with a more sensitive technique. In fact, one of these immunochemically negative hephroblastomas gave a fairly positive reaction when studied by immunofluorescence.

The metastases of nephroblastomas were negative in three cases, and positive in one (obtained at necropsy). These positive reactions were due to the same antigen (Fig. 2), which is thus present in almost all the Wilms' tumors. We propose to call it W antigen (W for Wilms).

By immunofluorescence we obtained the staining of the cytoplasm of tumor cells (Fig. 3), and of intercellular deposits by antiserum against W antigen. These patterns were not seen if the antiserum was previously absorbed out with sufficient amounts of a nephroblastoma extract rich in W antigen (i.e., 5 mg of protein per ml of antiserum for our richest saline extract).

*Control Studies.* W antigen was not found in saline or phytic extracts of normal kidney or in those of other normal organs. Absorption of antibodies against W antigen by these extracts was always inefficient.

W antigen seemed absent from fetal organs. We studied extracts of fetal kidney with special care. None of them reacted with or absorbed out the antinephroblastoma antisera. Furthermore these latter antisera did not stain frozen sections of fetal kidneys (generally metanephros, and in one case mesonephros) in immunofluorescence studies. Two fetal epididymis, taken as derivatives from Wolff's organ, were negative in immunofluorescence (their amount was much too small to allow immunochemical studies). The same negative reaction was observed with sections of fetal urinary bladder. All the other fetal organs were similarly negative by immunochemical methods. However, with regard to phytic extracts, only those of liver, lung, and digestive tract were used.

W antigen did not seem to be of infectious origin, as antiserum against W antigen reacted neither with extracts of the most common urinary microbes (Proteus, *E. coli*, Enterococcus, Staphylococcus) nor with Mycoplasmas; it did not stain smears of different strains of Mycoplasma (*Orale* I and II, *Pneumoniae*) in immunofluorescence. Furthermore, antisera against Mycoplasma *Orale* I and II, *Pneumoniae*, and *Salivarium* (Collindale, Great Britain) did not precipitate with nephroblastoma extracts.

Tumors of different organs rarely contained W antigen. Among the positive ones, all of the glandular type, we found one renal carcinoma, one pool of mammary carcinomas, and several samples of gastric origin: one cancer extract, one cancerous gastric juice, the bronchial mucus of a pulmonary metastasis. Obviously the location of the tumor did not seem to influence its positivity for W antigen.

*Absence of Relationship of W Antigen with Other Tumor-Associated Antigens.* There is no reaction between antibody against W antisera, and $\alpha$ fetoprotein, carcinoembryonic antigen of digestive tumors, carcinofetal glial antigen, $\alpha_2$ ferroprotein ($\alpha_2$H), and lactoferrin. Furthermore, antisera against these different antigens did not precipitate with W antigen.

Nephroblastoma extracts did not precipitate with a human serum rich in antibodies against Epstein–Barr virus (which precipitated with an extract of Burkitt cells), nor with a rabbit antiserum able to precipitate gs-3 antigen of RNA leukemogenic viruses.

*Chemical Nature of W Antigen.* The W antigen was soluble in phytic acid. It was destroyed by perchloric acid and altered by periodic acid. Its precipitin line was stained by the periodic acid–Schiff reagent and Alcian blue. The W antigen was unaltered by all the enzymes used, except insoluble Pronase, which weakened its precipitin line. It is thus likely that W antigen is a glycoprotein or perhaps a polysaccharide.

## DISCUSSION

We described in this paper a new human tumor-associated antigen. It was found to be different from all the other tumor antigens we tested. The only antigens we could not use for comparison were the fetal sulfoglycoprotein of Häkkinen (5) and the $\gamma$ fetoprotein described by Edynak (7), but these substances are obviously very different from our W antigen:

(*a*) Fetal sulfoglycoprotein is precipitable by cetylpyridinium chloride, and W antigen is not. Fetal sulfoglycoprotein is revealed in the cancerous gastric mucus with specific antisera by immunofluorescence, but antisera against W antigen did not stain this type of cancer mucus. (*b*) $\gamma$ Fetoprotein differs from W antigen by its electrophoretic mobility and its presence in fetal serum.

W antigen is not easily demonstrable, and it is present in small amounts in many nephroblastomas. It is thus understandable that Linder (12), in an immunochemical study on nephroblastomas, could not find any tumor-specific antigen. On the other hand, Wise *et al.* (8), who prepared antisera against nephroblastoma and absorbed them with normal kidney extract, obtained two precipitin lines with these antisera and the immunizing extracts. Little is known of these antigens, as their electrophoretic mobility and their repartition in adult, fetal, or cancerous organs have not been described. Other evidence of tumor-associated antigen(s) in nephroblastomas was obtained by Diehl *et al.* (13) in their study of cellular immunity in patients afflicted with this type of cancer.

W antigen was found in many, but not all, the nephroblastomas we studied. Negative results could be explained by a lack of sensitivity of the immunochemical methods. Obviously a more sensitive technique, such as radioimmunoassay, could have detected traces of W antigen in more nephroblastomas, and perhaps also in control organs, either adult of fetal.

It is remarkable that the presence of W antigen was shown in a few carcinomas that differed thoroughly from Wilms' tumors in their location and their histology. These positive cases were tumors of stomach, breast, and kidney. Considering this latter location, we must admit that we studied only a few

cases of adult-type kidney carcinomas. Although we found mostly negative results for W antigen in these carcinomas, we could not ascertain that this negativity is the rule. In previous work of this laboratory (14), the precipitin line of an antigen associated with adult kidney carcinomas was described, but the antigen was neither isolated nor characterized so we do not know if the precipitin line observed in that study has any relationship with W antigen.

The absence of W antigen from fetal organs is an important finding. It follows that W antigen seems not to be a carcinoembryonic antigen. To firmly establish this point, we studied fetal kidneys with special care. During embryonic and fetal life, three different kidneys appear successively and grow for a time. We had no difficulty in studying the metanephros, either by immunochemical methods or by immunofluorescence, and the negative results we obtained seemed, therefore, highly reliable. As for the mesonephros, we could only use immunofluorescence. As antisera against W antigen did not stain the one case of true mesonephros or fetal epidymis, which has the same embryologic origin, the absence of W antigen in mesonephros is likely. We could obviously not study the pronephros, but is seems quite improbable that this very small and shortlived organ could contain antigens not existing at least in the other fetal kidneys.

Although we cannot eliminate the possibility of detecting traces of W antigen in normal and (or) fetal organs by radioimmunological techniques, we can envisage as likely the hypothesis of a true neoantigen. It is possible then to speculate on its origin.

An attractive hypothesis would be that W antigen is synthesized under the action of an exogenous and possibly viral genome. We would then have to admit that the causative virus could cause cancer in more than one organ, as W antigen was found in a few cancers of other organs. Another possibility would be that W antigen is the label of a passenger virus, which has a special tropism for nephroblastomas. It is not possible at the present time to prove either hypothesis, but it is worthwhile to recall that viral-induced nephroblastomas have been described several times in animals. Lacour *et al.* (15) were able to create nephroblastomas in chickens by inoculating them with avian myeloblastosis virus. Other authors obtained nephroblastomas in hamsters that had received kidney hamster cells transformed by Simian virus 40 (16). These two types of virus are also able to induce tumors in organs other than kidney. These facts favor the hypothesis of a viral origin for human nephroblastomas.

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