Autoantibodies in Canine Neoplasms. I. Reactivity of Autologous 7 S γ-Globulins with Tumor Cells

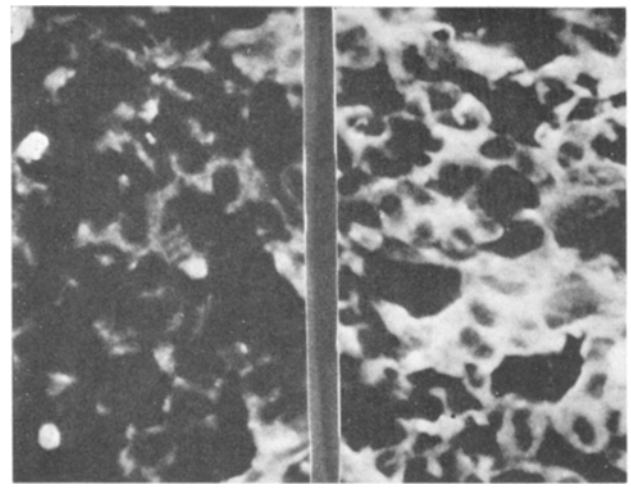
Many investigators have observed that not only do tumor cells lack certain antigens present in their normal counterparts but also have found numerous cancer-specific antigens[1]. In many instances, the tumor-bearing host responds to these antigens by eliciting a cellular and/or humoral immune response[2,3].

BLAKEMORE and MCKENNA[4] demonstrated by passive hemagglutination the presence of antibodies in the sera of patients with malignant melanomas and a rise in titer of antibodies when these patients were injected with extracts of tissues from humans who were similarly afflicted. BURTIN et al.[5] demonstrated reactivity to autologous tumor cells in the sera of humans with cancers of the stomach, breast and large intestine. This antigenic activity appeared to be associated with the microsomal fraction of the tumor cells. Using the antiglobulin consumption test, ANTHONY and PARSONS[6] have shown the presence of a 7 S γ-globulin on cancer cells. Although the role of these tumor-specific autoantibodies in the host is not known; many clinicians maintain that some patients do show clinical evidence of resistance to their tumors[7]. Recently, three dogs in the total of 40 tumorous canines studied to date were found to lack tumor autoantibodies.

In this study, the immunological response in 8 spontaneous canine neoplasms was investigated by both immunofluorescent and antiglobulin consumption techniques. Tumors studied were 3 fibrosarcomas, 2 mast cell sarcomas, 1 leiomyosarcoma, and 2 mixed mammary carcinomas. Dogs were bled prior to surgery. Their malignant tissues and normal counterparts were quick-frozen upon surgical removal and stored at −20 °C. For the immunofluorescent tests, each dog's normal and malignant tissues were cut in 4 μ serial sections on a cryostat unit and were air dried. Each serum was fractionated on a G-100 and G-200 series of Sephadex gel columns, and the fractions were conjugated to fluorescein isothiocyanate (FITC) by the method of NAIRN[8]. The conjugated globulins were purified on a G-25 Sephadex gel column and adsorbed with mouse liver powder to reduce nonspecific fluorescence. Goat anti-7 S canine globulin and anti-19 S canine globulin were also labeled. Normal and malignant tissue sections were reacted with autologous 19 S and 7 S labeled canine globulins as well as with labeled anti-19 S and anti-7 S canine globulins. Staining reactions were carried out in moist chambers at 37 °C followed by two 10 min washings in phosphate buffered saline (PBS), pH 7.2. Lissamine rhodamine B 200 dye conjugated to bovine serum albumin was used as a counterstain to aid in the prevention of nonspecific staining. Controls included FITC-tagged pooled normal dog sera concentrated 3 times and reacted with both normal and malignant tissue sections. Blocking reactions in which each unlabeled serum was added to a serial section prior to the addition of the identical labeled serum were also run. No reaction was considered specific unless it could be blocked by this technique. Sections mounted in a medium consisting of 9 parts PBS, pH 7.4, and 1 part reagent grade glycerol were examined by darkfield fluorescent microscopy using a Reichert Biozet Microscope with exciter filters BG 12 and UG 1 and with a Wratten 2 A (Kodak) barrier filter. A few slides were observed with a Leitz Routine Laboratory Phase-Fluorescence Microscope Model SM-M for simultaneous phase contrast and fluorescent viewing. Serial sections of the normal and tumor tissues were stained with hematoxylin and eosin and were compared with the fluorescent stained sections for cellular orientation.

For the antiglobulin consumption test, a 25% suspension of tissue, either normal or malignant, was made in anti-canine globulin of a 1:64 dilution. Incubation with occasional stirring was carried out at room temperature for 15 min. The mixture was centrifuged for 30 sec in an Adam's Sero-fuge and the supernatant fluid separated from the tissue residue. The residue was washed 5 times in PBS, pH 7.2, incubated in 0.4 ml of PBS, pH 7.2, at 56 °C for 45 min with occasional stirring, and centrifuged for 5 min in an Adam's Sero-fuge. The supernatant fluids from both the absorption and elution procedures were titrated against a sensitized system consisting of a 3% suspension of A' canine erythrocytes in autologous serum. These erythrocytes had been incubated for 30 min at 37 °C with a 1:5 dilution of canine anti-A serum, centrifuged at 1000 rpm in a clinical centrifuge and washed 4 times in 2 ml quantities of PBS, pH 7.2.

In all 8 cases studied, the 7 S globulin fraction of each tumor dog's serum was found to react with his own malignant cells although the serum from one dog with a mammary carcinoma reacted only very weakly with autologous tumor cells. No reactivity between normal cells and FITC-labeled autologous 7 S globulin was observed nor was there any reactivity between FITC-labeled 19 S globulin fractions and either normal or malignant tissues. Labeled normal canine globulins did not react with either normal or malignant tissue sections. Malignant, but not normal, cells also stained with tagged anti-7 S canine globulins but not with anti-19 S canine globulins as seen in the Figure.



Typical fluorescent stain and blocking reaction. Top-mixed mammary tumor illustrating specific fluorescence with FITC-ACG. Bottom-serial section illustrating blocking of the above reaction by prior incubation with unlabeled ACG. × 630.

[1] E. D. DAY, *Immunochemistry of Cancer* (Charles C. Thomas Publishers, Springfield, Ill. 1965).
[2] I. HELLSTROM and K. HELLSTROM, Proc. natn. Acad. Sci., USA *60*, 1231 (1968).
[3] B. HATTLER and B. AMOS, Monogr. Surg. Sci. *3*, 1 (1966).
[4] W. BLAKEMORE and J. MCKENNA, Surgery St. Louis *52*, 213 (1962).
[5] P. BURTIN, S. VON KLEIST, W. RAPP, F. LOISILLIER, A. BONATTI and P. GRABAR, *Immunopathology*, IVth International Symposium (Schwabe and Co. Publishers, Basle 1966), p. 91.
[6] H. ANTHONY and M. PARSONS, Nature *206*, 275 (1965).
[7] C. SOUTHAM, Fedn Proc. *24*, 1007 (1965).
[8] R. NAIRN, *Fluorescent Protein Tracers* (Williams and Wilkins Co., Baltimore, Md. 1964).

Results of the antiglobulin consumption tests performed on 6 of the 8 sets of dog tissues substantiated the immunofluorescent observations. All 6 malignant tissues were found to adsorb anti-canine globulin; i.e., completely remove it from the supernatant fluid except in the case of 1 mammary tumor (the same one which gave the weak fluorescent reaction) which reduced the titer in the supernatant fluid from 32 to 8. Normal tissue counterparts of malignant tissues lacked the ability to adsorb anti-canine globulin; i.e., the titer in the supernatant fluids was either identical to, or within one dilution of, the titer of unadsorbed anti-canine globulin. Furthermore, normal tissues did not release any antiglobulin in the elution procedure as did the malignant tissues.

Although one cannot say with certainty that the globulins found on the tumor cells by the indirect fluorescent technique and by antiglobulin consumption tests are specific for tumor antigens on the surface of malignant cells (they could be normal globulins nonspecifically associated with the cell membrane), their correlation with the specific 7 S immunoglobulin in the sera leads one to believe that they are specific. These results are in direct contrast to those of STIRLING et al.[9] who observed no specific fluorescence of tumor cells from 24 human breast carcinomas when treated with an FITC-tagged anti-human globulin. However, they have been substantiated in our laboratory by CONGDON[10] who noted specific staining in 4 malignant canine tumors and KRAMER[11] who found immunochemical differences between the plasma membrane fractions of canine tumor tissues and their normal counterparts.

Zusammenfassung. Durch Immunofluoreszenz wird eine spezifische Bindung autologer γ-Globuline an neoplastische Zellen des Hundes nachgewiesen.

L. E. YURKO[12], N. J. BIGLEY and G. P. WILSON III

The Academic Faculty of Microbial and Cellular Biology, The Ohio State University, Columbus (Ohio 43210, USA), 28 March 1969.

[9] G. STIRLING, E. DAOUD and L. HUGHES, Nature *201*, 1235 (1964).
[10] A. CONGDON, M. Sc. Thesis, Ohio State University, Columbus, Ohio (1967).
[11] H. KRAMER, M. Sc. Thesis, Ohio State University, Columbus, Ohio (1968).
[12] Supported, in part, by a National Science Foundation fellowship.

Autoantibodies in Canine Neoplasms. II. Tumor Tissue Specificity and Lack of Cross-Reactivity with Embryonic Antigens

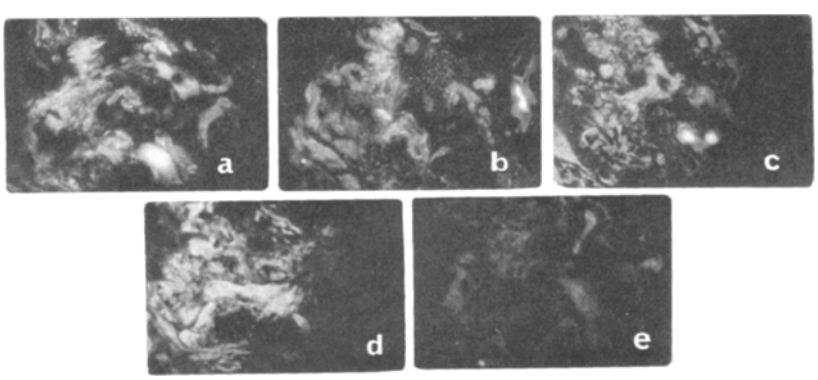
The cross-reactivity of antibodies produced in response to tumor antigens is variable. In man, tissue specific tumor antibodies[1], systems-specific tumor antibodies[2], and universal tumor antibodies have been described. Antibodies to some tumor antigens also react with embryonic antigens[2–4] whereas others do not[5].

An earlier study of 8 canine malignancies showed that canines react to their tumor tissues by producing a 7 S γ-globulin specific for the tumor tissue but not the corresponding normal tissue[6]. The degree of cross-reactivity of this globulin with other canine tumors of the same type, with canine tumors of various other types, and with embryonic canine tissues was examined and is reported here.

Dogs were bled prior to surgery and the globulin fraction of their sera separated by passage through a G-100 Sephadex gel column. Malignant tissues and normal counterparts from each animal were quick-frozen immediately upon surgical removal and stored at $-20\,^{\circ}C$. Tumors studied were: 5 mammary carcinomas, 3 mastocytomas, and 2 squamous cell carcinomas. Embryos in the second and third trimesters of gestation were obtained by Caesarean section and longitudinal sections of them were quick-frozen immediately upon delivery.

The methods used to conjugate the globulin fractions of each dog's serum to fluorescein isothiocyanate (FITC), to purify the conjugate, and to treat tissues with these immunofluorescent stains were described previously[6]. The FITC-tagged globulin fraction of each dog's serum was reacted with cryostat-cut sections of the dog's own

[1] P. BURTIN, S. VON KLEIST, W. RAPP, F. LOISILLIER, A. BONATTI and P. GRABAR, *Immunopathology*, IVth International Symposium (Schwabe and Co. Publishers, Basel 1966), p. 91.
[2] P. GOLD and S. FREEDMAN, J. Expl Med. *122*, 467 (1965).
[3] V. KOLMYKOVA and A. EROSHKINA, Probl. Oncol. *5*, 2 (1959).
[4] L. HIRSCHFELD, W. HALBER and I. ROSENBLAT, as quoted by V. KOLMYKOVA and A. EROSHKINA, Probl. Oncol. *5*, 2 (1959).
[5] J. MCKENNA, R. SANDERSON and W. BLAKEMORE, Cancer Res. *24*, 754 (1964).
[6] L. YURKO, N. J. BIGLEY and G. WILSON, Experientia *25*, 1087 (1969).



Tumor group specificity of fluorescent reaction. Serial sections of mammary carcinoma M_1 stained with: (a) FITC-labeled sera from M_1; (b) FITC-labeled sera from M_2; (c) FITC-labeled sera from M_5; (d) FITC-labeled sera from M_4; (e) unlabeled serum from M_1 + FITC-labeled serum from M_1. Note positive fluorescence in (a) through (d) and the lack of fluorescence in (e). × 400.