J. Immunol. **117**, 1762 (1976); A. G. Romualdez, Jr., and P. A. Ward, in *Membranes and Neoplasia: New Approaches and Strategies*, V. T. Marchesi, Ed. (Liss, New York, 1976), p. 65; W. Orr, J. Varani, P. A. Ward, *Am J. Pathol.*, in press.
7. L. G. Raisz, *J. Clin. Invest.* **44**, 103 (1965); C. L. Trummel, G. R. Mundy, L. G. Raisz, *J. Lab. Clin. Med.* **85**, 1001 (1975).
8. N. Ruddle of Yale University School of Medicine provided this line.
9. G. R. Mundy, J. Varani, W. Orr, M. D. Gondek, P. A. Ward, *Nature (London)* **275**, 132 (1978).
10. R. A. Willis, *The Spread of Tumours in the Human Body* (Butterworth, London, 1973), p. 231.
11. A. E. Postlethwaite, J. M. Seyer, A. H. Kang, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 871 (1978).
12. K. Ushijima, H. Nishi, A. Ishikura, H. Hayashi, *Virchows Arch. B* **21**, 119 (1976).
13. E. Viadana, I. D. J. Bross, J. W. Pickren, *Br. J. Cancer* **27**, 336 (1973).
14. Micropore filters with different porosities were used to compare the migration of tumor cells and neutrophils. A larger pore size is needed for chemotactic studies of monocytes and macrophages than for neutrophils because of the greater volume and relative nondistensibility of the nuclei of the former cells. When filters with 12-μm pores are used to study locomotion of neutrophils, massive and rapid cell migration in the filters obscures differences in random and chemotactic movement.
15. W.O. is a fellow of the R. Samuel McLaughlin Foundation, Toronto. G.R.M. is the recipient of American Cancer Society faculty research award FRA-148. Supported by grant CH-69B from the American Cancer Society, contract NO1-CB-63980 from the National Cancer Institute, and NIH grants AM 21584 and AI 09651.
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14 August 1978; revised 26 September 1978

Tumor Surveillance: How Tumors May Resist Macrophage-Mediated Host Defense

Abstract. *Both normal human serum and supernatant from explanted malignant tumors contained a heat-stable low-molecular-weight factor that inhibited monocyte activation in vitro. In contrast, serum from individuals with solid tumors enhanced monocyte activation. It is suggested that the systemic activation of monocytes that occurs in malignant disease may be an appropriate host response but that successful tumors may continue to grow because they subvert the normal physiological signal for inhibition of macrophage activation.*

A considerable body of evidence shows that cells of the mononuclear phagocyte series can recognize and kill transformed (*1*) and malignant (*2*) cells, and a defensive role in host surveillance against neoplastic disease has been ascribed to the macrophage (*3*). Investigations of monocyte or macrophage function in tumor-bearing hosts have shown that the capacity to mobilize macrophages into inflammatory sites may be defective (*4*) and that the chemotactic responses of monocytes may be depressed (*5*). Moreover, studies in both animal (*6*) and human (*7*) systems indicate that this depression of monocyte function is mediated by tumor products. Snyderman and his colleagues have argued that by this mechanism tumors may escape macrophage-mediated surveillance (*8*).

Monocytes and macrophages possess membrane receptors for the Fc portion of immunoglobulin G (Fc receptors) (*9*), the functional expression of which is markedly increased during cellular activation in vivo (*10, 11*) and in vitro (*11*). Changes in the expression of this receptor can be conveniently assayed to provide a reliable indicator of cellular activation or depression (*11–13*). The peripheral blood monocytes of individuals with solid malignant tumors (*14*) and lymphomas (*15*) exhibit an increased expression of Fc receptors and this change does not occur readily in a variety of nonmalignant diseases (*14*). Experiments in guinea pigs suggest that macrophage activation is controlled by an inhibitory factor in normal serum (*11*). We therefore wished to look for factors in human serum and tumor supernatants that might be modulating macrophage function. Here we show that the activation of normal human monocytes in vitro is inhibited by a factor in normal human serum but enhanced by serum from individuals with solid malignant tumors. We show that tumors release a factor resembling that present in normal serum, which inhibits monocyte activation in the same manner.

Fresh serum samples were obtained from untreated patients with primary carcinomas of the lung or colon. The majority were free of obvious infections and metastatic disease. Control serums were obtained from normal donors of comparable age. No age- or sex-related differences in monocyte function or in serum effects on monocyte function were observed. Studies of soluble tumor products in vitro were performed largely on freshly excised superficial bladder carcinomas because of the discrete nature of the tumor mass, its sterility, and the dissociability of the cells, and because normal mucosa from an adjacent area could be easily identified and sampled by transurethral resection. Squamous cell carcinomas of the lung and adenocarcinomas of the breast were also studied.

Normal mononuclear cells were isolated from defibrinated blood by centrifugation over a Ficoll-Triosil gradient. The cells were washed twice in Hanks balanced salt solution (BSS) and resuspended at a concentration of 2×10^6 per milliliter in RPMI 1640 containing 20 percent heat-inactivated fetal calf serum (FCS). A 1-ml portion was placed in each chamber of dual tissue culture slides (Lab-Tek Products). After incubation at 37°C for 1 hour, the nonadherent cells were removed by washing the monolayer with serum-free medium. The presence of 20 percent serum during monocyte adherence is important because it prevents the adherence of lymphocytes. Platelets were removed in the defibrination procedure. Approximately 95 percent of the adherent cells at this stage are monocytes. After 24 hours of culture, contaminating lymphocytes and granulocytes, identified by Giemsa staining, were reduced to less than 2 percent.

Samples of tumor tissue and normal bladder mucosa were washed and gently minced with scissors in cold RPMI 1640 containing antibiotics. The suspension was adjusted to 10 percent by volume and cultured for 24 hours at 37°C, after which cell-free supernatants were obtained by centrifugation. In some experiments a single cell suspension was obtained by pressing the tissue through a fine steel mesh. Adherent cells were then depleted by allowing the cells to settle onto glass for 90 minutes, after which nonadherent cells were collected by aspiration. Ultrafiltration of supernatants was performed by using cone filters with more than 95 percent retention for molecules larger than 50,000 or 25,000 daltons (Amicon Centriflo membranes, type CF50A or CF25), following the manufacturer's instructions. Ultrafiltration of fresh serum samples was performed in the same way. Heat inactivation was carried out by incubating ultrafiltrates at 56°C for 35 minutes. Normal monocytes that adhered under the uniform conditions described were then cultured in RPMI 1640 containing antibiotics supplemented with the serums or supernatant preparations at the concentrations specified. No significant differences in cell loss or viability were observed. After culture, the cells were washed in serum-free BSS and the assay for Fc receptor expression was performed in BSS in the absence of any serum. A panel of ox erythrocytes bearing increasing amounts

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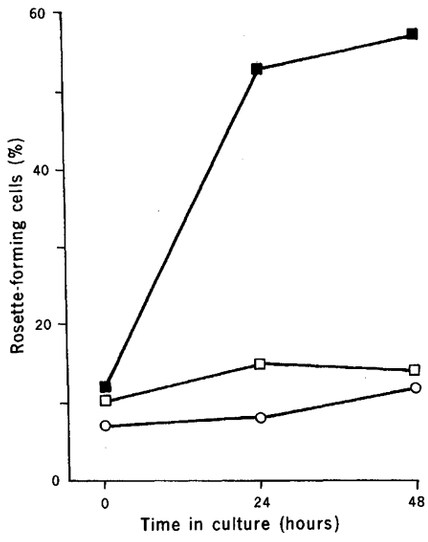

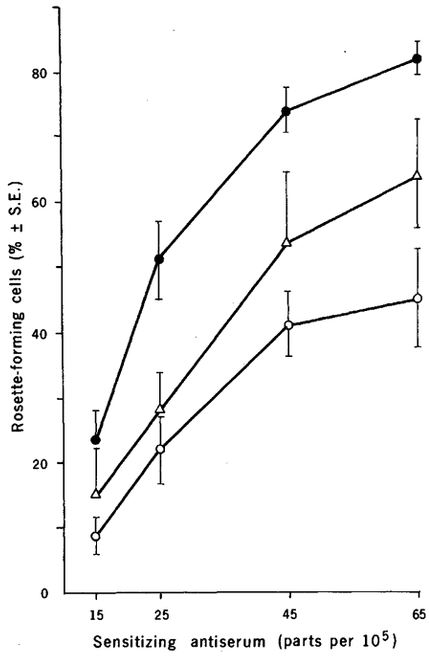

Fig. 1 (left). Percentage of rosette-forming monocytes as a function of time in culture, using erythrocytes sensitized with a constant suboptimal dose of antiserum (batch R300). Normal monocytes were cultured in RPMI 1640 in the presence of (■) heat-inactivated FCS, (○) fresh normal (autologous) human serum, or (□) an ultrafiltrate of fresh normal human serum, all at concentrations of 20 percent. The medium and serum supplement were renewed at 24 hours. Values are means of four experiments. Fig. 2 (right). Percentage of rosette-forming monocytes as a function of the degree of erythrocyte sensitization (antiserum batch R300) after 24 hours of culture in RPMI 1640 containing an ultrafiltrate of (○) fresh normal human serum or (●) fresh tumor bearer's serum, both at a concentration of 20 percent, or in (△) serum-free conditions. We compared 14 patients with 14 normal donors; values are expressed as means ± standard errors.

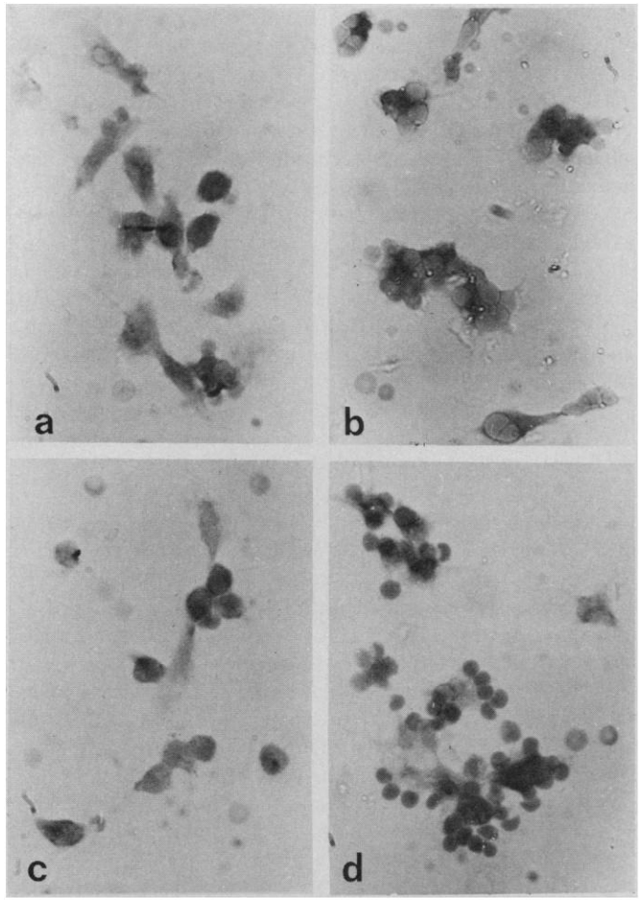

Fig. 3. (a and b) Same population of normal monocytes cultured in RPMI 1640 containing an ultrafiltrate of either fresh normal human serum (a) or fresh serum from a donor with squamous cell carcinoma of the bronchus (b), assayed after 24 hours with the same preparation of sensitized erythrocytes. (c and d) Same population of normal monocytes cultured in RPMI 1640 containing 10 percent FCS either with (c) or without (d) tumor cell supernatant at 20 percent, assayed after 24 hours with the same preparation of sensitized erythrocytes.

of specific antibody was used in a rosette assay as previously described (*14*).

Normal monocytes cultured for 48 hours in the presence of 20 percent FCS exhibited a striking increase in the expression of Fc receptors, but this change did not occur in the presence of 20 percent fresh autologous serum or an ultrafiltrate of fresh normal serum containing nothing larger than 50,000 daltons (Fig. 1). This resembles the behavior of guinea pig macrophages under comparable conditions (*11*). The behavior of monocytes in serum-free conditions was then compared with that of monocytes cultured in the presence of ultrafiltrates of fresh serum either from normal donors or from donors with solid tumors. Receptor expression was assayed after 24 hours, since the data in Fig. 1 show that significant increases in receptor expression can occur over this period. The increase in receptor expression occurring in serum-free conditions was consistently inhibited by an ultrafiltrate of normal human serum, but consistently enhanced by an ultrafiltrate of serum from individuals with solid malignant tumors (Figs. 2 and 3).

The effects of tumor supernatants on monocyte function were tested in the same way, except that normal monocytes were cultured in RPMI containing FCS at a final concentration of 10 percent. Cultures were supplemented with supernatant or ultrafiltrates of supernatant or serum, as specified in Fig. 4 at concentrations of 20 percent. After 24 hours the culture medium was removed and the cells were washed three times. The rosette assay was performed in the usual way in serum-free BSS. Both tumor supernatant and fresh normal serum, but not normal tissue supernatant, contained a heat-stable activity of less than 25,000 daltons that inhibited the activation of normal monocytes occurring in the presence of 10 percent FCS during 24 hours of culture (Figs. 3 and 4). No decrease in inhibitory activity was observed in supernatants from tumor cells depleted of adherent cells. No inhibition was observed with supernatants from the peripheral blood mononuclear cells of tumor bearers. Tumor supernatants at a concentration of 10 percent produced a lesser but often significant degree of inhibition (data not shown).

These data show that fresh normal human serum contains a factor (or factors) that inhibits the activation, as judged by increased Fc receptor expression, of human monocytes in vitro. Such a factor is also present in guinea pig serum acting on guinea pig peritoneal macrophages.

The latter is heat-stable but labile over long periods of storage and appears to be consumed or exhausted by macrophages in vitro (*11*). The data indicate that the systemic activation of monocytes, as judged by increased Fc receptor expression in individuals with solid malignancies (*14*), is mediated by a change in serum components in that normal monocytes can be induced to differentiate into the kind of monocyte found in tumor bearers by the presence of tumor bearer's serum. Since these effects are mediated by ultrafiltrates, the molecular weight of the factors involved must be less than 50,000. The addition of whole serum from tumor bearers to normal monocytes was avoided because of the potential blocking or activating effects of alloantibodies specific for monocyte antigens. In experiments where monocytes were cultured in the presence of whole serum only autologous serum was employed.

The activation of monocytes in the presence of tumor bearer's serum cannot be entirely accounted for by a decreased level of inhibitor because the degree of activation that occurs is greater than that occurring in serum-free conditions, indicating the presence of a positive signal for activation. Such a signal might be produced by lymphokines; the molecular weight of human macrophage activating factor, for example, which seems to be equivalent to migration inhibition factor (*16*), is less than 50,000 (*17*). Other positive signals for activation in vivo need not depend on a specific interaction between lymphocytes and antigen.

The extensive studies on chemotaxis, particularly those of Snyderman and colleagues, have clearly shown that circulating monocytes in tumor bearers exhibit depressed chemotactic function even in early disease (*5*, *7*). In a previous study (*14*) and in the work reported here, using the functional expression of Fc receptors and the subsequent ingestion of immune complexes (*13*) as an indicator, we have found enhanced peripheral function. These two, and possibly other, distinct functions of macrophages may well be modulated independently. Other workers have found enhanced lysis of antibody-coated target cells by monocytes from tumor bearers (*18*), and studies of nitroblue tetrazolium reduction indicate an enhanced monocyte response to phagocytic stimuli in midterm disease and a depressed response in late disease (*19*).

Studies on macrophages at tumor sites in murine systems have shown that these cells are activated (*12*, *20*), whereas some studies in humans indicate depressed macrophage function (*21*). However, the status of macrophages at tumor sites, within the potential range of depression or activation, is presumably determined by a balance of antagonistic signals. Both chemotaxis assays (*8*) and the present assay detect a factor produced by tumors that inhibits monocyte function and may permit the growth of the tumor. Such factors might account for the continued growth of tumors in spite of the presence of large numbers of infiltrating host macrophages (*22*). In the work reported here the inhibitory factor produced by tumors resembles an inhibitory factor in normal serum. In a direct comparison, both fresh normal serum and tumor supernatant contained an activity of less than 25,000 daltons that inhibited the activation of monocytes over the same period, was heat-stable, and overrode any stimulatory activity resulting from the presence of 10 percent FCS.

On the basis of these data we suggest that while the systemic activation of monocytes, mediated by changes in small serum components, may be an appropriate host response, one of the reasons why tumors continue to grow is that the normal physiological inhibitor of macrophage activation is produced within the tumor mass. In this case the macrophage-mediated host response would exert a selective pressure in favor of tumors producing the inhibitor. Possible candidates for such an inhibitory factor are not completely elusive: for example, insulin exerts an inhibitory effect on macrophage function in vitro (*23*) and hormones of unknown function with insulin-like activity are present in normal serum (*24*) and are also secreted by tumor cells (*25*).

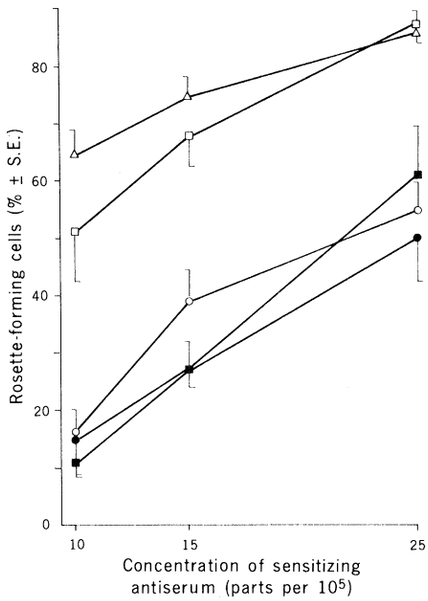

Fig. 4. Percentage of rosette-forming monocytes as a function of the degree of erythrocyte sensitization (antiserum batch R302) after 24 hours of culture of RPMI 1640 containing 10 percent FCS and (△) no supplement, (□) normal bladder cell supernatant at a concentration of 20 percent, (■) tumor cell supernatant at a concentration of 20 percent, (●) a heat-inactivated ultrafiltrate of tumor cell supernatant containing nothing larger than 25,000 daltons at a concentration of 20 percent, and (○) a heat-inactivated ultrafiltrate of fresh normal human serum containing nothing larger than 25,000 daltons at a concentration of 20 percent. Data represent 12 tumors and six normal serums.

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References and Notes

1. J. B. Hibbs, Jr., *Science* **180**, 868 (1973).
2. ———, L. H. Lambert, J. S. Remington, *Nature (London) New Biol.* **235**, 48 (1972); W. H. Churchill, W. F. Piessens, C. A. Sulis, *J. Immunol.* **115**, 781 (1975); G. A. Currie, *Nature (London)* **273**, 758 (1978).
3. M. H. Levy and E. F. Wheelock, *Adv. Cancer Res.* **20**, 131 (1974); G. A. Currie, *Biochim. Biophys. Acta Rev. Cancer* **458** (CR3), 134 (1976).
4. S. A. Eccles and P. Alexander, *Br. J. Cancer* **30**, 42 (1974); S. J. Normann and E. Sorkin, *J. Natl. Cancer Inst.* **57**, 135 (1976).
5. D. A. Boetcher and E. J. Leonard, *J. Natl. Cancer Inst.* **52**, 1091 (1974); R. Snyderman, M. C. Pike, L. C. Altman, *Ann. N.Y. Acad. Sci.* **256**, 386 (1975).
6. R. Snyderman and M. C. Pike, *Science* **192**, 370 (1976); S. J. Normann, *J. Natl. Cancer Inst.* **60**, 1091 (1978); R. M. Fauve, B. Henri, H. Jacob, J. A. Gaillard, F. Jacob, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4052 (1974); M. Nelson and D. S. Nelson, *Immunology* **34**, 277 (1978).
7. R. Synderman, L. Meadows, W. Holder, S. Well, Jr., *J. Natl. Cancer Inst.* **60**, 737 (1978).
8. M. C. Pike and R. Snyderman, *J. Immunol.* **117**, 1243 (1976).
9. A. F. LoBuglio, R. S. Cotran, J. H. Jandl, *Science* **158**, 1582 (1967); H. Huber and H. H. Fudenberg, *Int. Arch. Allergy Appl. Immunol.* **34**, 18 (1968); A. Berken and B. Benacerraf, *J. Exp. Med.* **123**, 119 (1966).
10. W. P. Arrend and M. Mannik, *J. Immunol.* **110**, 1455 (1973).
11. J. Rhodes, *ibid.* **114**, 976 (1975).
12. R. S. Kerbel, *Nature (London)* **259**, 226 (1976).
13. L. E. Spitler, A. S. Levin, D. P. Stites, H. H. Fudenberg, H. Huber, *Cell. Immunol.* **19**, 201 (1975); S. I. Hamburg, R. E. Manejias, M. Rabinovitch, *J. Exp. Med.* **147**, 593 (1978). Rosettes were formed at room temperature over a 1-hour period and both bound and ingested erythrocytes were scored. Over longer periods at 25°C the majority of erythrocytes were phagocytized. The determination of erythrocyte phagocytosis yielded the same results as the determination of erythrocyte binding.
14. J. Rhodes, *Nature (London)* **265**, 253 (1977).
15. A. F. LoBuglio, *J. Lab. Clin. Med.* **76**, 888 (1970).
16. J. R. David and H. G. Remold, in *Immunobiology of the Macrophage*, D. S. Nelson, Ed. (Academic Press, New York, 1976), p. 401.
17. R. E. Rocklin, H. G. Remold, J. R. David, *Cell. Immunol.* **5**, 436 (1972); L. H. Block, H. Jaksche, S. Bamberger, G. Ruhenstroth-Bauer, *J. Exp. Med.* **147**, 541 (1978).

18. R. E. Nyholm and G. A. Currie, *Br. J. Cancer* 37, 337 (1978).
19. D. W. Hedley and G. A. Currie, *ibid.*, p. 747.
20. J. S. Haskill, J. W. Proctor, Y. Yamamura, *J. Natl. Cancer Inst.* **54**, 387 (1975).
21. J. L. Boak and T. C. Agurunobi, in *The Macrophage and Cancer*, K. James, W. McBride, A. Stuart, Eds. (James McBride and Stuart, Edinburgh, 1977), p. 418; J. Rhodes and N. Plowman, in preparation.
22. R. Evans, *Transplantation* **14**, 468 (1972); R. S. Kerbel and H. F. Pross, *Int. J. Cancer* **18**, 432 (1976).
23. J. Rhodes, *Nature (London)* **257**, 597 (1975); R. S. Bar, C. R. Kahn, H. S. Koren, *ibid.* **265**, 632 (1977); R. H. Muschel, N. Rosen, O. M. Rosen, B. R. Bloom, *J. Immunol.* **119**, 1813 (1977).
24. O. Oelz, E. R. Foresch, H. F. Bunzli, R. E. Humbel, W. J. Ritschard, in *Handbook of Physiology*, vol. 1, *Endocrinology*, D. F. Steiner and N. Frienkel, Eds. (Williams & Wilkins, Baltimore, 1972), p. 685.
25. T. Hyodo, K. Kegyesi, C. R. Kahn, J. P. McLean, H. G. Friesen, *J. Clin. Endocrinol. Metab.* **44**, 1175 (1977); J. E. DeLarco and G. J. Todaro, *Nature (London)* **272**, 356 (1978).
26. We thank N. M. Bleehen, N. Plowman, D. Rapp, D. K. C. Cooper, and F. Howlett and his staff for cooperation. Supported by the Cancer Research Campaign of Great Britain.

17 August 1978

Oocyte-Follicle Cell Gap Junctions in *Xenopus laevis* and the Effects of Gonadotropin on Their Permeability

Abstract. *Junctions between* Xenopus laevis *oocytes and follicle cells have been identified as gap junctions by the passage of microinjected fluorescent dye from oocytes to follicle cells. The opening or assembly of these junctions, or both, appears to be regulated by gonadotropins.*

The functional relation between the amphibian oocyte and the single layer of follicle cells that surrounds it is not well understood. Follicle cells have been implicated in a number of gonadotropin-regulated processes involved with amphibian oocyte growth and development. These include steroidogenesis (*1*), initiation of yolk protein (vitellogenin) uptake (*2*), and increases in amino acid uptake and protein synthesis in the oocyte (*3*). Ultrastructural examination of the ovary of *Xenopus* has shown that the follicle cells possess numerous macrovilli which project through the vitelline envelope and contact the oocyte surface, forming junctional complexes of an unidentified nature with the oocyte membrane (*4*). Recently we have observed, with the aid of lanthanum tracers, that there are small gap junctions between the follicle cell macrovilli and the oocyte in *Xenopus* (Fig. 1A). Gap junctions have also been identified in the mammalian ovary between granulosa cells and oocytes (*5*). In this report we present evidence that the junctional complexes between the amphibian oocyte and its follicle cells are gap junctions and that these junctional complexes may be hormonally regulated.

Gap junctions, through their structural modifications of the membranes of adjointed cells allow for cell to cell communication by the passage of ions and of molecules of low molecular weight (*6*). To determine whether the junctional complexes observed between the amphibian oocyte and its surrounding follicle cells are gap junctions, we isolated complete follicles (*4*) from *Xenopus* ovaries and injected approximately 50 nl of the fluorescent dye, 6-carboxyfluorescein through the follicular wall into the oocytes. Fluorescein and its derivatives pass freely through gap junctions, but do not readily permeate nonjunctional membranes (*6*). The injected dye was allowed to diffuse through the ooplasm for 1 to 2 hours. Smaller injection volumes or shorter diffusion times result only in lower levels of fluorescence in responding oocyte-follicle cell complexes. Two hours after injection the oocytes in their follicles were transferred to a solution of 1 m*M* phenylarsine oxide in a double-strength salt solution [OR 2 (*7*)]. Phenylarsine oxide, a sulfhydryl agent, stiffens the theca, the follicle cell layer, and the acellular vitelline envelope, while the hypertonic solution causes the oocyte to shrink slightly and separate from the theca and the follicle cell layer. This separation allows the two layers to be easily removed, one after the other, with watchmaker's forceps. Very often the vitelline envelope and the follicle cell layer are removed as a single unit. For our purposes this technique proved to be superior to the standard means for removing follicle cells which involves placing dissected oocytes in Ca^{2+}-Mg^{2+}-free or EDTA-containing media (*8*, *9*).

Follicle cell layers were examined from stage IV, V, and VI oocytes (*10*). Oocytes were taken from unstimulated animals and from animals that had been stimulated with 1000 I.U. of human chorionic gonadotropin (hCG) (*11*) 24 hours previously. Ten healthy females were stimulated with hCG. Nearly all follicle cell layers removed from the fluorescein-injected oocytes of the ten stimulated females showed fluorescence, indicative of the passage of a dye through a gap junction (Fig. 1, B and C). Fluorescence was observed in the follicle cell layers from oocytes of all stages examined. Normally not all of the follicle cells from the same oocyte fluoresced with the same intensity, although occasionally all were equally fluorescent. Neither uninjected oocytes enclosed in their intact follicles nor dissected oocytes enclosed only in follicle cells and exposed directly in fluorescein-containing medium cause fluorescence in follicle cells.

In some *Xenopus* ovaries, follicles containing two oocytes are present. Although these oocytes are enclosed within

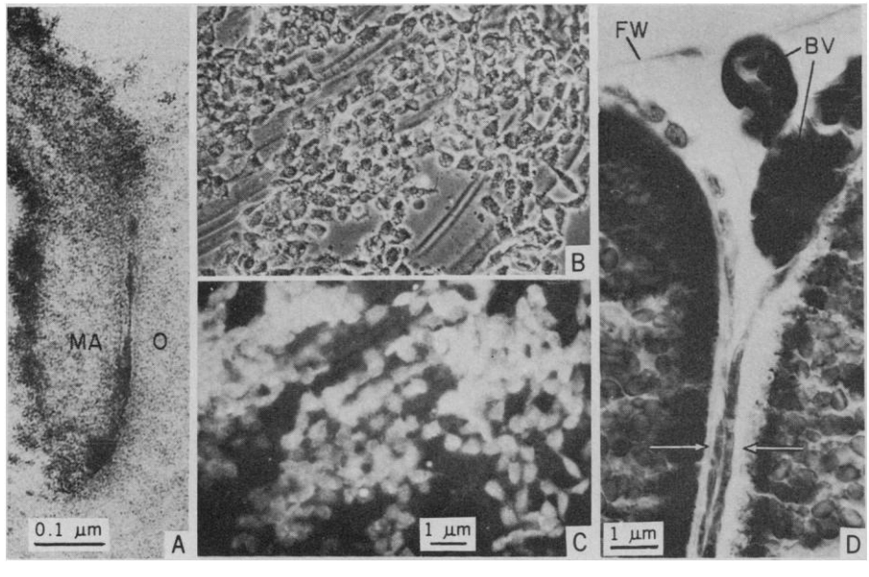

Fig. 1. (A) A small gap junction between a follicle cell macrovillus (*MA*) and an oocyte (*O*) demonstrated by means of a lanthanum tracer. (B) Phase-contrast and (C) fluorescent microscope images of the same field of follicle cells from an oocyte injected with 6-carboxyfluorescein from an hCG-stimulated animal. (D) Two oocytes enclosed in a common follicle (*FW*, follicular wall) but having individual follicle cell layers (arrows); *BV*, blood vessels in theca.

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