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Production of Factors with Immunosuppressive Activity by a Murine Lymphoblastoid Tumor Cell Line[1] (39667)

JOHN M. MANSFIELD, WILLIAM M. SHANNON,[2] SHING-ERH YEN, AND JOHN H. WALLACE

Department of Microbiology and Immunology, School of Medicine, University of Louisville, Louisville, Kentucky 40201

The multiple interfaces that exist between cells of the immune system and cells of progressively growing tumors are currently the subject of much interest. Not only are the tumor-destructive or tumor-inhibitory functions of T and B lymphocytes and accessory cells being critically examined, but also various immune-escape mechanisms of tumor cells are being elucidated.

The balance that exists, or the relationship, between a host and its tumor frequently may be weighted in favor of tumor growth, as opposed to immune elimination of the tumor cells. For example, the spontaneous release of tumor-specific antigen by tumor cells promotes the "blocking" and triggering of T cells at sites anatomically remote from the tumor (1-4), the formation of blocking antigen-antibody complexes (5-7), and the appearance of immune complex-associated tissue pathology (3). Furthermore, tumor cells have been shown to secrete factors which possess immunoregulatory properties. A well-documented example is the secretion of immunosuppressive products by Ehrlich's ascites tumor cells *in vivo* (8-11). Similarly, other tumor cell lines secrete or release antiinflammatory factors (12), factors which inhibit macrophage cell function (13), or factors which affect specific T or B lymphocyte functions (14, 15).

In our laboratories we have been interested in immunologic mechanisms which contribute to tumor cell protection, and the relationships between immunosuppressive factors secreted by tumor cells of lymphoid origin and factors secreted by normal T and B lymphocytes. We have previously described the secretion by a murine lymphoblastoid tumor cell line (L1210) of several lymphokine-like substances *in vitro* (16). In the present study we describe the release of a lympholine-like factor from an isolated clone of L1210 cells, and the immunosuppressive effects of culture-derived fluids containing such factors.

Methods and materials. Animals. Inbred DBA/2 ♂ and C57B1/6 ♂ mice (18-20 g) and Hartley ♂ albino guinea pigs (0.6 kg) were obtained from Lab Supply Co., Indianapolis, Indiana. All animals were maintained in AAALAC-approved facilities and were handled according to NIH guidelines.

Tumor cells. L1210 lymphocytic leukemia cells (strain LEO8G39) were originally obtained from the Southern Research Institute, Birmingham, Ala. The tumor cell line has been routinely passaged as an ascites tumor in DBA/2 mice, and has also been maintained *in vitro* as a suspension culture in RPMI-1640 medium, pH 7.2, containing 10% heat-inactivated fetal calf serum and 50 μg/ml Gentamicin. All cells were incubated at 37° in a 5% CO_2 in air atmosphere. The clone of cells (L1210/A3) used in the present study was selected as an isolated colony from a culture medium-agarose plate (17), and was subsequently recloned three times at a low cell density in agarose in order to ensure monoclonality. The L1210/A3 cells have since been maintained *in vitro* in suspension culture.

Tumor cell products. L1210/A3 cells were washed in serum-free medium and subsequently recultured in serum-free medium at a cell density of 5×10^6 cells/ml for a period of 24 hr at 37° in a 5% CO_2 in air atmosphere. Following the incubation period, cells were separated from the culture

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[2] Present address is the Southern Research Institute, Birmingham, Alabama.

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supernatant fluids (SF) by centrifugation and membrane filtration (0.45 μm pore size). SF were stored frozen (−70°) or were exhaustively dialyzed against distilled water, lyophilized, and stored at 4°; lyophilized SF materials were reconstituted to the concentration desired in phosphate-buffered saline (PBS; pH 7.2) or medium. Protein determinations of SF were done by conventional procedures.

Lymphokine assays. Supernatant fluids were incubated *in vitro* with syngeneic (DBA/2), allogeneic (C57B1/6), or xenogeneic (guinea pig) peritoneal macrophages (PEC) in tests for macrophage migration inhibitory factor (MIF) activity. PEC were harvested from the peritoneal cavities of animals which had received an intraperitoneal injection 5 days earlier of 0.5 ml (mice) or 15 ml (guinea pig) sterile Marcol 52 (Humble Oil Co.). After washing, PEC were packed into plugged capillary tubes which were then cut at the cell-fluid interface. Cell-packed tubes were loaded into Sykes-Moore chambers in which control fluids or medium containing SF were placed (18); serum supplementation of medium containing SF for the MIF tests was 10% fetal calf serum for mouse PEC and 10% guinea pig serum for guinea pig PEC. After a 24–48 hr incubation period at 37°, the areas of PEC emigration from capillary tubes were measured by planimetry, and the migration inhibition values (MI)[3] were calculated. Tests for other lymphokine-like activities in SF were performed as detailed elsewhere (18), in comparison with appropriate controls.

Immunization and SF injection regimens. DBA/2 mice were injected intraperitoneally or intravenously with SF in 0.1 ml PBS prior to, at the same time as, or after, the intraperitoneal injection of 3×10^8 washed sheep erythrocytes (SRBC; in 0.5 ml PBS). For *in vitro* immunization procedures, pooled nonimmune DBA/2 spleen cells were cultured in 35 × 10 mm tissue culture dishes at a concentration of 6×10^6 viable cells in 2.0 ml medium (EHAA, containing 10% fetal calf serum, 5×10^{-5} *M* 2-mercaptoethanol, and 50 μg/ml Gentamicin) at 37°, 5% CO_2 in air for 4 days according to the procedure of Click *et al.* (19); 0.1 ml EHAA containing 2×10^7 washed SRBC, with or without SF, was added to cultures at the beginning of the culture period. Some control animals and *in vitro* spleen cell cultures received an amount of bovine serum albumin (BSA) equivalent to the amount of SF administered to test animals or cultures.

Plaque-forming cell (PFC) assays. Spleen cells from immunized mice, or spleen cells from the primary *in vitro* antibody response cultures, were assayed for PFC responses specific for SRBC following immunization. The Kennedy and Axelrad modification (20) of the Jerne plaque technique was used. Briefly, SRBC monolayers were prepared in 60 × 15 mm Corning tissue culture plates by adding erythrocytes to poly-1-lysine treated plates; spleen cells, from immunized mice or the *in vitro* cultures, were subsequently plated over the SRBC monolayers in PBS containing guinea pig serum as a source of complement. Plates were incubated at 37° for 1 hr in order to develop the plaques, which were then read macroscopically. Indirect (IgG) PFC were enumerated with the aid of developing antiserum (rabbit antimouse IgG, heavy chain specific; Cappel Laboratories).

Results. Lymphokine assays. Both frozen and lyophilized-reconstituted L1210/A3 culture fluids (SF) exhibited macrophage migration inhibitory factor (MIF)-like activity *in vitro* with normal syngeneic, allogeneic, or xenogeneic peritoneal macrophage cells (Table I); lysates of L1210/A3 cells did not possess significant MIF-like activity (ie. MI values of ≥80). The MIF-like activity associated with L1210/A3-SF was not due to cytotoxicity since excessively high concentrations (5000 μg/ml) failed to alter the

TABLE I. MACROPHAGE MIGRATION INHIBITORY FACTOR-LIKE ACTIVITY OF L1210/A3 CULTURE SUPERNATANT FLUIDS (SF) *in Vitro*.

Peritoneal exudate cell source	SF Migration inhibition values (± SE)[a]
DBA/2 mice (H-2^d)	38 (±5)
C57BL/6 mice (H-2^b)	43 (±3)
Guinea Pig	25 (±7)

[a] SF concentrations = 50 μg/ml; positive controls for all experiments were pooled lots of guinea pig lymphocyte culture fluids containing MIF.

[3] MI = [(migration of PEC in SF preparations)/(migration of PEC in cuture medium)] × 100.

viability of peritoneal macrophages during a 48 hr incubation period. The MIF-like activity was demonstrable with SF preparations containing as little as 25 μg protein/ml. The specific absence of other lymphokine-like activities, such as proliferation-inhibition factor, chemotactic factors, lymphotoxin, interferon, and blastogenic factor, was determined by incubation of appropriate target cells with SF in comparison with positive controls of antigen or mitogen stimulated guinea pig lymphocyte culture fluids.

Immunosuppressive effects of SF. The injection of SF in amounts of $\geq$50 μg up to 3 days prior to or on the same day as SRBC injection resulted in a subsequent suppression of splenic PFC responses to SRBC (Fig. 1); both the IgM and IgG responses were significantly suppressed. Injection of SF by either the intravenous or intraperitoneal routes resulted in immunosuppression. SF from L1210/A3 cultures were also capable of suppressing the splenic PFC responses of non-H-2 related mice (eg., C57B1/6) to SRBC. In contrast to the effects of pretreatment of mice with SF, the administration of SF after SRBC immunization caused no changes in the splenic PFC responses to SRBC. The addition of SF to spleen cell cultures undergoing a primary *in vitro* antibody response to SRBC resulted in suppression of the resultant day four PFC responses (Fig. 2); the *in vitro* suppressive effects,

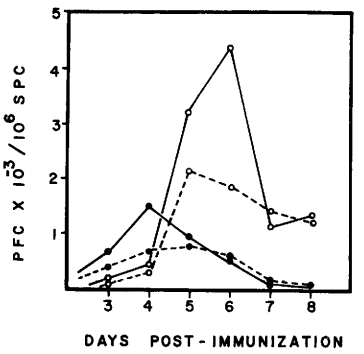

FIG. 1. Immunosuppressive effects of MIF-containing supernatant fluids (SF) from L1210/A3 cultures on the splenic plaque-forming cell (PFC) responses of DBA/2 mice to SRBC. (——) controls; 50 μg BSA ip 3 days prior to SRBC immunization. (- - -) 50 μg SF ip 3 days prior to SRBC. *P* values for peak control responses compared to peak experimental responses are <0.005 for IgM (●) and <0.001 for IgG (○).

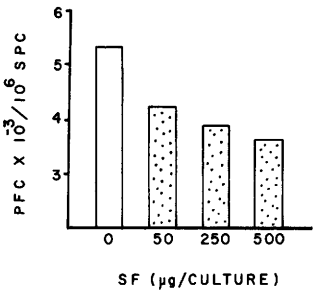

FIG. 2. Immunosuppressive effects of L1210/A3 culture supernatant fluid (SF) preparations on the primary *in vitro* day 4 antibody response of DBA/2 spleen cells to SRBC. The *P* values for suppressor activity of SF preparations of 250 μg/culture or greater are <0.01.

however, were not as marked as the *in vivo* effects, and only the higher concentrations of SF produced significant suppression. SF did not affect the viability of mouse spleen cells *in vitro* at any concentration tested.

Discussion. The L1210 cell is a weakly antigenic, rapidly proliferating lymphoblastoid tumor cell which arose in DBA/2 mice as the result of exposure to methylcholanthrene (21-23). It does not resemble a mature T or B lymphocyte in that it does not possess either membrane-associated Thy 1.2 (θ) antigen or immunoglobulin (24); although this mouse tumor cell does not have C3b receptors on the membrane, several strains of the L1210 cell are reported to possess an Fc receptor for IgG (25). Thus, whether the L1210 cell more closely resembles an immature lymphocyte or a null cell is open to question.

The present study demonstrates that a clone of L1210 cells (L1210/A3) spontaneously releases an MIF-like substance *in vitro* which has macrophage migration inhibitory properties in the conventional MIF test for syngeneic, allogeneic, and xenogeneic peritoneal macrophages. The MIF-like activity of the L1210/A3 culture supernatant fluid (SF) preparations was not due to a toxic effect of SF, as determined by dye-exclusion tests, for macrophages or other cells. This study further demonstrates that L1210/A3 culture SF possessing MIF activity suppress the primary *in vivo* and *in vitro* plaque-forming cell (PFC) responses by syngeneic mouse spleen cells to SRBC; although immunosuppression was statistically

significant *in vivo* and *in vitro*, the *in vitro* responses were less marked. We have found previously that ascites fluid from tumor-bearing DBA/2 mice possesses both the MIF and immunosuppressive properties, but the source of such factors (i.e., tumor cells or host lymphoid cells) remains in doubt (unpublished observations).

Whether the MIF-like factor(s) present in L1210/A3 culture SF preparations are also responsible for the immunosuppressive activity, or whether separate "suppressor factors" are present, is under investigation at this time. Preliminary experiments have shown that both the MIF-like material and suppressor activity cannot be separated by conventional column chromatography techniques; the immunobiologically active fraction has a mol wt in the range of >20,000 to <60,000 daltons.

The relationship of L1210-derived immunosuppressive factors to such factors secreted by other tumor cells (26–30) or, indeed, by normal lymphoid suppressor cells (31) remains to be determined. Several reports link MIF-like activity with an antigen nonspecific suppressor factor secreted by appropriately-stimulated murine lymphocytes (32, 33). The "site" of action of L1210/A3 culture SF may be on the afferent portion of the immune response since administration of SF after antigen exposure does not cause suppression. Also, SF preparations do not suppress the blastogenic responses of murine T or B cell populations *in vitro* to mitogens or antigen (i.e., SF does not possess other lymphokine-like activities such as proliferation inhibitory factor or interferon). Since an effect on macrophages has been demonstrated, SF may alter antigen distribution *in vivo* or antigen uptake *in vitro* by such cells, and may thereby cause the observed suppression. In any event, the effects of SF preparations on specific macrophage functions in both T-dependent and T-independent immune responses must be examined in order to test this hypothesis.

Summary. A clone of L1210 cells has been shown to spontaneously release an MIF-like factor *in vitro*. Culture supernatant fluids containing such MIF activity are immunosuppressive when injected with or prior to an antigenic stimulus in syngeneic mice, and are also suppressive to a lesser degree when administered with antigen in a primary *in vitro* antibody response system. The secretion of immunosuppressive factor(s) by L1210 cells may represent yet another example of tumor cell associated mechanisms which alter host immune system function.

1. Currie, G. A., and Basham, C., Brit. J. Cancer **26,** 247 (1972).
2. Thomson, D. M. P., Sellens, V., Eccles, S., and Alexander, P., Brit. J. Cancer **28,** 377 (1973).
3. Poskitt, P. K. F., Poskitt, T. R., and Wallace, J. H., J. Exp. Med. **140,** 410 (1974).
4. Currie, G., Biochim. Biophys. Acta **458,** 135 (1976).
5. Sjogren, H. O., Hellstrom, I., Bansal, S. C., and Hellstrom, K. E., Proc. Nat. Acad. Sci. U.S.A. **68,** 1372 (1971).
6. Baldwin, R. W., Price, M. R., and Robins, R. A., Nature New Biol. **238,** 185 (1972).
7. Baldwin, R. W., Bowen, J. G., and Price, M. R., Brit. J. Cancer **28,** 16 (1973).
8. McCarthy, R. E., Coffin, J. M., and Gates, S. L., Transplant **6,** 737 (1968).
9. Hrsak, I., and Marotti, T., Eur. J. Cancer **9,** 717 (1973).
10. Yamazaki, H., Nitta, K., and Umezawa, H., Gann **64,** 83 (1973).
11. Hrsak, I., and Marotti, T., J. Nat. Cancer Inst. **53,** 1113 (1974).
12. Fauve, R. M., Hevin, B., Hedwig, J., Gaillard, J. A., and Jacob, F., Proc. Nat. Acad. Sci. U.S.A. **71,** 4052 (1974).
13. North, R. J., Kirstein, D. P., and Tuttle, R. L., J. Exp. Med.**143,** 559 (1976).
14. Wells, J. H., Cain, W. A., Wells, R. S., and Bozalis, J. R., Int. Arch. Allergy **47,** 362 (1974).
15. Hersh, E. M., and Drewinko, R., Cancer Res. **34,** 215 (1974).
16. Mansfield, J. M., Wallace, J. H., and Shannon, W. M., Fed. Proc. **32,** 3670 (1973).
17. Macpherson, I. *In* "Tissue Culture Methods and Applications." Academic Press, 276, (1973).
18. Bloom, B. R., and Glade, P. R. (Eds.). *In Vitro* Methods in Cell-Mediated Immunity, 578 p. Academic Press, N.Y., (1971).
19. Click, R. E., Benck, L., and Alter, B. J., Cell. Immunol. **3,** 264 (1972).
20. Kennedy, J. C., and Axelrad, M. A., Immunology **20,** 253 (1971).
21. Law, L. W., Dunn, T. B., Boyle, P. J., and Miller, J. H., J. Nat. Cancer Inst. **10,** 179 (1949).
22. Glynn, J. P., Humphreys, S. R., Trivers, G., Bianco, A. R. and Goldin, A., Cancer Res. **23,** 1008 (1963).
23. Bonmassar, E., Bonmassar, A., Vadlamudi, S., and Goldin, A., Cancer **27,** 1356 (1971).
24. Shevach, E. M., Stobo, J. D., and Green, I., J.

Immunol. **108,** 1146 (1972).
25. Cooper, S. M., and Sambray, Y., J. Immunol. **117,** 511 (1976).
26. Kateley, J. R., Kamo, I., Kaplan, G., and Friedman, H., J. Nat. Cancer Inst. **53,** 1371 (1974).
27. Poste, G., Cancer Res. **35,** 2558 (1975).
28. Poste, G., Exp. Cell Res. **92,** 283 (1975).
29. Werner, D., and Schulte, M., Eur. J. Cancer **11,** 521 (1975).
30. Wong, A., Mankovitz, R. and Kennedy, J. C., Int. J. Cancer **13,** 530 (1974).
31. Pierce, C. W., and Kapp, J. A., Contemp. Top. Immunobiol. **5,** 91 (1976).
32. Tadakuma, T., Kuhner, A. L., Rich, R. R., David, J. R., and Pierce, C. W., J. Immunol. **117,** 323 (1976).
33. Tadakuma, T., and Pierce, C. W., J. Immunol. **117,** 967 (1976).

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