Multifaceted Evaluation of Human Tumor Immunity Using a Salt Extracted Colon Carcinoma Antigen[1] (36650)

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Research during the past decade has indicated that most, if not all, human tumors possess tumor-associated antigens (1). This is based on *in vivo* and *in vitro* immunologic test systems which use intact tumor cells (2, 3), tumor cell homogenates (4) and subcellular fractions obtained by sonic disruption, enzyme digestion, and ultracentrifugation (5–7) as the source of antigen. There is also one report of a soluble tumor-associated antigen found in the fluid of a cystic melanoma (8).

Ideally, the use of soluble tumor antigens will not only eliminate a number of technical difficulties entailed in preserving and storing tumor cells, but will also enable the investigator to study patients sequentially in relation to their response to treatment. This soluble antigen, if shown to be tumor specific and immunogenic, may also be useful for specific immunotherapy. Recent success in salt extraction of soluble HL-A antigens (9) has led investigators to apply similar methods to the extraction of tumor-associated antigen from chemically induced hepatoma in guinea pigs (10). The antigenic activity of the extracted material was determined by the skin reactions in immunized and nonimmunized animals. This report presents preliminary evidence that human tumor-associated antigen can be separated by salt extraction and can be used in a multifaceted immunological evaluation of the cancer patient.

Materials and Methods. The patient, a 60 year old white male, had undergone resection of primary adenocarcinoma of the colon in 1962. Local recurrences were resected in 1964, 1966, 1968, and 1970. Radiation therapy or chemotherapy were not given. In November, 1971, he presented with a left abdominal mass. At laparotomy, a mucinous tumor was found to involve the spleen with multiple omental deposits. Splenectomy was done and the histological diagnosis was mucinous adenocarcinoma, metastatic to the spleen.

Extraction of Tumor Antigen. A single cell suspension was prepared from the fresh tumor tissue (obtained at laparotomy) by scraping, teasing and sieving through a 60-mesh screen into Medium 199 containing 50 μg streptomycin and 50 U of Penicillin/ml. Red blood cells were lysed by tris-buffered ammonium chloride for 15 min (11), and 5×10^9 tumor cells with 40% viability by trypan blue dye exclusion (12) were used for antigen extraction. The method previously described by Meltzer *et al.* (10), was used with slight modifications. Fifty ml of 3 *M* KCL made in phosphate-buffered saline at pH 7.2 were added to the tumor cells in a 25 × 200 mm screw-cap glass tube. This equilibrated on a rocker at 4°, 4×10^4 g for 16 hr. The contents were then centrifuged at 0° for 60 min. The slightly viscid supernatant was transferred into cellulose tubing and dialyzed against 20 vol of deionized water for 1 hr. This was followed by dialysis against 20 vol of phosphate-buffered saline for 24 hr with change of solution every 8 hr. To achieve rapid equilibrium, all dialyses were performed on a rocker at 4°. The dialyzed extract was again centrifuged at 0° and at 40,000*g* for 30 min. The supernatant was concentrated by pervaporation. The concentrated extract was then mixed with an equal volume of 4 *M* ammonium sulphate to precipitate the protein.

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After 1 hr of equilibration in the cold room, the precipitate was centrifuged at 40,000*g* at 0° for 30 min. Supernatant was discarded and the precipitate redissolved in 5 ml of phosphate buffered saline, pH 7.2. It was then dialyzed against phosphate buffered saline for 4 hr. Protein concentration was estimated by measurement of optical density at a wavelength of 280 nm (OD 280). This final preparation was sterilized by passing it through a 0.45 μ Millipore filter (Millipore Corporation, Bedford, Massachusetts) and was kept in the refrigerator at 4°.

Lymphocyte Cultures. Lymphocyte cultures were prepared and stimulated as previously described (13). Venous blood was defibrinated by swirling with glass beads, the red blood cells sedimented with dextran and the resultant white cell rich serum adjusted to 10^6 lymphocytes per ml. Each culture contained 10^6 patient's or normal donor's lymphocytes, 1 ml of patient's or normal donor's heat-inactivated sera and 2 ml of minimal essential media. Individual cultures were stimulated with .05 ml of phytohemagglutinin-M (PHA) (Difco Laboratories, Detroit, Michigan), 10^6 allogeneic lymphocytes irradiated with 4,000 R (MLC) and with the extracted tumor antigen in a dose range of 20–1000 μg/ml of culture. Cultures were harvested after 3 days of incubation at 37° in air atmosphere containing 5% CO_2. Mixed lymphocyte cultures were harvested after 7 days. During the last 8 hr of incubation, 1 μCi of [^{3}H]thymidine (Schwarz BioResearch, Orangeburg, New York; sp act 1.9 Ci/m*M*) was added to the cultures. The blastogenic responses were determined by the incorporation of the isotope into the acid insoluble fraction as measured by liquid scintillation counting and expressed as counts per minute per 10^6 lymphocytes (cpm).

Leukocyte Migration Inhibition. Inhibition of leukocyte migration was measured as described by Wolberg (4) with slight modification. Sixty ml of venous blood were defibrinated by swirling with glass beads. The white cell rich serum was diluted with 3 vol of normal saline and layered on top of a Hypaque–Ficoll gradient (14) [10 parts of 33.9% Hypaque (Winthrop Laboratories, New York, N.Y. mixed with 24 parts of 9% Ficoll (Pharmacia Fine Chemicals, Uppsala, Sweden)] in siliconized 25 × 150 mm centrifuge tubes. Red blood cells and granulocytes were sedimented through the gradient by centrifugation at 500*g* for 40 min. The leukocyte layer at the interface of the plasma and the Hypaque–Ficoll containing approximately 80% lymphocytes, 10% monocytes and 10% granulocytes and a few red blood cells was removed, washed twice with Medium 199 and resuspended in Medium 199 at concentration of 5×10^7/ml. Ten lambda capillary pipettes (Drummond) were filled with the cell suspension, sealed at one end with clay (Clay–Adams), and centrifuged at 150*g* for 8 min. They were then broken at the cell–medium interface. The portion containing the packed leukocytes was inserted into the thin end of a siliconized 9 in. Pasteur pipette already filled with Medium 199, with or without extracted tumor antigen, in a dose range of 20–1000 μg/ml. This end was sealed with clay and broken 2 in. beyond the inner tube. This break was sealed with stopcock grease (Dow–Corning). After 20 hr of incubation in a horizontal position at 37° and in air atmosphere containing 5% CO_2, the distance of migration along the Pasteur pipette was determined under the microscope with a submillimetric grid. Each experimental point was determined in 6 replicate capillaries. The mean distance of migration was used to calculate the migration index as follows: migration index = mean distance of migration with tumor antigen divided by mean distance of migration of control. The migration index in the control solutions (Medium 199 alone) is thus 1.0. An index of 0.8 or less indicated significant inhibition of leukocyte migration.

Skin Testing. To detect delayed hypersensitivity, 0.1 ml of the extracted tumor antigen was injected intradermally in the original concentration of 6.7 mg/ml and also after 1:10 dilution. The patient was also tested with a battery of established delayed hypersensitivity skin test antigens. Induration was measured at 24 and 48 hr, and was recorded as the average diameter of two right-angle measurements in millimeters.

TABLE I. Blastogenic Responses of Patient's and Normal Donor's Lymphocytes to Various Mitogens.

tor	Counts per minute per 10^6 patient's lymphocytes				Counts per minute per normal donor's lymphoc	
	One week after surgery		Three weeks after surgery		Same time as first patient	
	Patient's serum	Normal serum	Patient's serum	Normal serum	Normal donor's serum	Pati
ulated	644	766	112	111	209	
emagglutinin	836	1756	40468	48522	149856	1
geneic lymphocytes[a]	11858	21302	4570	5422	8493	
antigen 20 μg/ml	596	792	—	—	326	
antigen 40 μg/ml	—	—	—	—	162	
antigen 60 μg/ml	—	—	3174	6034	—	
antigen 80 μg/ml	664	2698	—	—	228	
antigen 100 μg/ml	—	—	4960	14034	—	
antigen 170 μg/ml	704	1066	—	—	317	
antigen 330 μg/ml	478	1260	—	—	299	
antigen 600 μg/ml	—	—	162	170	—	
antigen 1000 μg/ml	—	—	46	332	—	

he first patient's study, normal donor's lymphocytes were used as stimulators and at the same time patient's lymphocytes were u
he same donor's lymphocytes. In the second patient's study, the stimulator lymphocytes were taken from another donor.

Results. The stock solution of the tumor antigen that was used in the various tests contained 6.7 mg/ml of protein and was bacteriologically sterile. Blastogenic response of the patient's lymphocytes to the extracted tumor antigen was determined 1 week and 3 weeks after surgery (Table I). In the first study a significant blastogenic response to the tumor antigen was detected only in the presence of normal donor's serum. The MLC also showed the suppressive effect of the patient's serum. In addition, the response to PHA was poor in both patient and normal serum, suggesting a general defect in the patient's lymphocyte function.

In the second study the response to PHA had recovered and the tumor antigen stimulated a more vigorous degree of blastogenesis. Only a slight inhibitory effect of the patient's serum was still present. In both studies, the low dose of tumor antigen (60–100 μg/ml) gave the most significant blastogenic responses and inhibition was noted at higher doses. Blastogenic response to tumor antigen was not detected among normal donor's lymphocytes although their response to PHA and to the patient's lymphocytes was adequate and a serum effect was not observed. The migration of patient's leukocytes was significantly inhibited by the tumor antigen (Table II). Maximum inhibition of migration was demonstrated with antigen concentration of 1,000 μg/ml. No inhibition was demonstrated with antigen concentrations lower than 330 μg/ml. The migration of the normal donor's leukocytes was not inhibited. One week after surgery, the patient showed delayed hypersensitivity skin reactions to dermatophytin-O among the established delayed hypersensitivity skin-test antigens. At this time, his reaction to the tumor antigen was negative. However, 3 weeks after surgery a positive delayed hypersensitivity skin reaction was observed and confirmed by a punch biopsy (Fig. 1). There was 10 mm induration to 0.67 mg and 6 mm to 0.067 mg. The normal subject was not skin tested.

TABLE II. Leukocyte Migration Test—Migration Indices of Patient's and Normal Donor's Leukocytes with Various Antigen Concentrations.

Antigen concentration	Patient's leukocytes	Normal leukocytes
Control	1.0	1.0
100 μg/ml	0.9	0.98
330 μg/ml	0.75	1.05
1000 μg/ml	0.45	1.42

Discussion. The biopsy proven demonstration of delayed hypersensitivity and the patient-specific inhibition of *in vitro* leukocyte migration to KCL-solubilized tumor antigen, strongly indicate that we have indeed detected cell-mediated tumor immunity in this patient. The interpretation of the patient-specific blastogenic response to the tumor antigen is somewhat more complex. Cell-associated allogeneic histocompatibility antigens are recognized as foreign and stimulate blastogenic response among lymphocytes that have never previously been exposed to them (15). On the other hand, individuals respond to soluble histocompatibility antigens only after prior exposure and sensitization (16, 17), although one report suggests that the latter is not needed (18). This is also true for soluble bacterial antigens (19).

The poor blastogenic response of patient's lymphocytes to the tumor antigen one week postsurgery can be accounted for by relative immunological incompetence as indicated by the poor response to PHA and to allogeneic lymphocytes and the inhibitory serum effect. Three weeks postsurgery, a vigorous response to the tumor antigen was associated with recovery of the immunological competence. Blastogenesis was stimulated with relatively low doses of tumor antigen (60–100 μg/ml) and doses higher than 330 μg/ml were inhibitory.

The most striking finding in this study was the failure of the normal donor's lymphocytes to respond to the tumor antigen. A related finding was previously reported by Jehn *et al.* (8), who demonstrated *in vitro* blastogenic response to soluble melanoma antigen by lymphocytes from melanoma patients but not from normal subjects. This is in contrast to our previous observation that whole tumor cells almost invariably stimulate blastogenesis among allogeneic normal donor's lymphocytes (20–22). The latter reac-

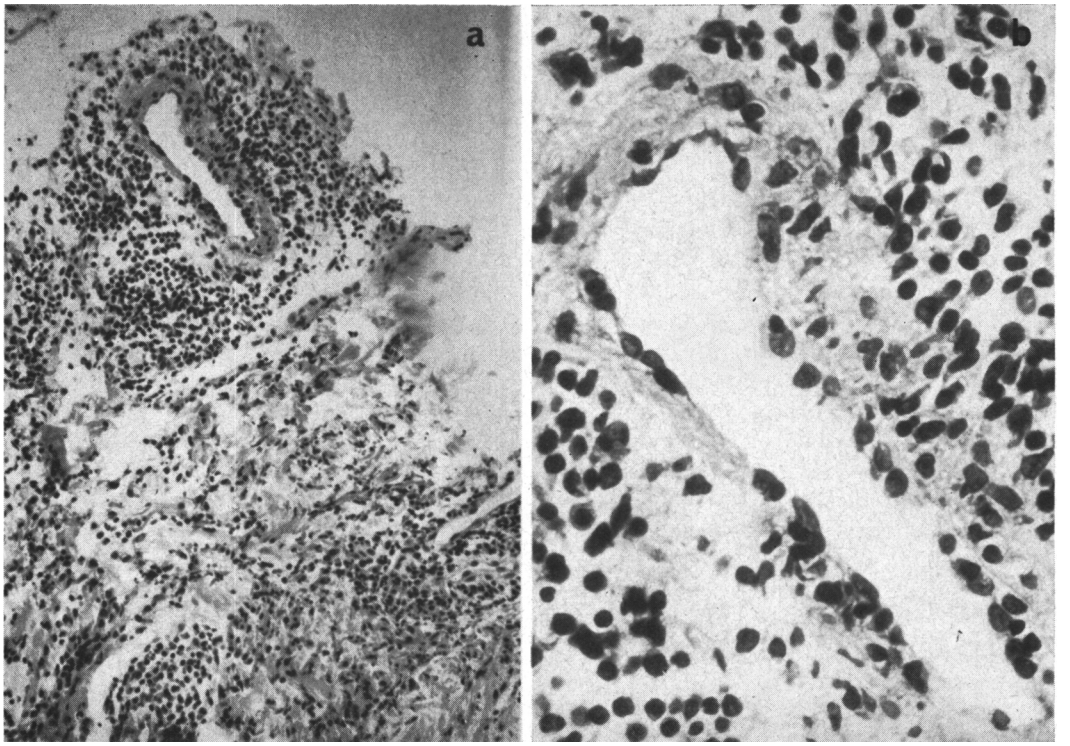

FIG. 1. Histological section from a skin biopsy, 24 hr after intradermal injection of tumor antigen. Mononuclear cell infiltration around blood vessels is consistent with delayed hypersensitivity reaction. (H &E, original magnification ×86—A); (H &E, original magnification ×344—B).

tions could be to tumor-associated or to HL-A antigens on the cell surface. Since the KCL method extracts both tumor and HL-A antigens, this confirms that allogeneic lymphocytes cannot respond to these antigens in soluble form without prior sensitization.

In conclusion, we suggest the following hypothesis: Peripheral blood lymphocytes can mount a primary blastogenic response *in vitro* to foreign antigens, only if the latter are cell-associated or particulate. In a soluble form, the same antigens do not stimulate a primary blastogenic response. They will, however, stimulate presensitized lymphocytes. Thus, our patient's lymphocytes responded to the tumor antigen because they were presensitized by the tumor *in vivo*. The normal donor's lymphocytes failed to respond to either tumor-associated or HL-A antigens in the soluble form because they were not presensitized. Therefore, in the multifaceted evaluation of cancer patients for specific tumor immunity, lymphocyte blastogenic response is a valuable adjunct to delayed cutaneous hypersensitivity and inhibition of leukocyte migration, provided soluble tumor antigen is used.

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1. Hersh, E. M., Gutterman, J. U., and Mavligit, G., *in* "Major Problems in Childhood Cancer" (Carl Pochedly, ed.). 1972 (In Press).

2. Hellstrom, I., Hellstrom, K. E., Sjogren, H. O., and Warner, G. A., Int. J. Cancer **7**, 1 (1971).

3. Nagel, G. A., St. Arneault, G., Holland, J. F., Kirkpatrick, D., and Kirkpatrick, R., Cancer Res. **30**, 1828 (1970).

4. Wolberg, W. H., Cancer Res. **31**, 798 (1971).

5. Hollinshead, A., Glew, D., Bunnag, B., Gold, P., and Herberman, R., Lancet **1**, 1191 (1970).

6. Oren, M. E., and Herberman, R. B., Clin. Exp. Immunol. **9**, 45 (1971).

7. Viza, D., Davies, D. A. L., and Harris, R., Nature (London) **227**, 1249 (1970).

8. Jehn, U. W., Nathanson, L., Schwartz, R. S., and Skinner, M., New Engl. J. Med. **283**, 329 (1970).

9. Reisfeld, R. A., Pellegrino, M. A., and Kahan, B. D., Science **172**, 1134 (1971).

10. Meltzer, M. S., Leonard, E. J., Rapp, H. J., and Borsos, T., J. Nat. Cancer Inst. **47**, 703 (1971).

11. Boyle, W., Transplantation **6**, 761 (1968).

12. Evans, H. M., and Schulemann, W., Science **39**, 443 (1914).

13. Hersh, E. M., Harris, J. E., and Rogers, E. A., J. Reticuloendothel. Soc., **7**, 567 (1970).

14. Thorsby, E., and Bratlie, A., *in* "Histocompatibility Testing" (P. I. Terasaki, ed.). Williams and Wilkins, Baltimore (1970).

15. Bach, F., and Hirschhorn, K., Science **143**, 813 (1964).

16. Kahan, B. D., Reisfeld, R. A., Epstein, L. B., and Southworth, J. G., *in* "Histocompatibility Testing" (E. S. Curtoni, P. L. Mattiuz, and R. M. Tosi, eds.). Williams and Wilkins, Baltimore (1967).

17. Leventhal, B. G., Mann, D. L., and Rogentine, G. N., Jr., Transplantation Proc. **3**, 243 (1971).

18. Viza, D. C., Degani, O., Dausset, J., and Davies, D. A. L., Nature (London) **219**, 704 (1968).

19. Pearmain, G., Lycette, R. R., and Fitzgerald, P. H., Lancet, **1**, 637 (1963).

20. Mavligit, G., Hersh, E. M., and McBride, C., Proc. Amer. Ass. Cancer Res. (Abstract) **13**, 67 (1972).

21. Gutterman, J. U., McCredie, K. B., and Hersh, E. M., Proc. Amer. Ass. Cancer Res. (Abstract) **13**, 80 (1972).

22. Anderson, R. J., McBride, C. M., and Hersh, E. M., Cancer Res. **32**, 988 (1972).

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