[CANCER RESEARCH 33, 2373-2377, October 1973]

Impaired Responsiveness of Lymphocytes and Serum-inhibitory Factors in Patients with Cancer[1]

Nicole Suciu-Foca, Joseph Buda, James McManus, Traute Thiem, and Keith Reemtsma

Department of Surgery, College of Physicians and Surgeons, Columbia University, New York 10032 [N. S.-F., J. B., T. T., K. R.], and French and Polyclinic Medical School, New York, New York 10001 [J. M.]

SUMMARY

Cellular immune responsiveness, as measured by lymphocyte transformation in one-way mixed-leukocyte cultures and in phytohemagglutinin (PHA)-stimulated cultures, was evaluated in 60 patients with cancer and in 81 normal controls. The effect produced by sera from these subjects on *in vitro* transformation of lymphocytes was tested on autologous cells and on homologous responding cells from a constant panel of 10 healthy volunteers.

The responsiveness of lymphocytes from cancer patients to PHA and to a battery of mitomycin-treated allogeneic cells was significantly lower than that of normal controls. Lymphocyte reactivity in the presence of autologous cancer serum was inversely related to the extent of the disease. Pooled normal serum enhanced the responsiveness of lymphocytes from cancer patients but had no such effect on control cultures from healthy subjects. Sera from cancer patients reduced the PHA and mixed-leukocyte culture responsiveness of normal panel lymphocytes to a level that was significantly lower than that found in the presence of sera from healthy controls. The degree of inhibition produced by cancer sera on the blastogenic response of normal lymphocytes increased with advanced disease. Suppression of lymphocyte transformation was markedly greater in mixed-leukocyte cultures than in cultures exposed to PHA.

Out of 60 cancer sera investigated, 23 possessed *HL-A* reactivity against a random panel of lymphocytes. Our findings demonstrate that serum-inhibitory factors are at least partially responsible for impairment of T-cell responsiveness in cancer patients. It is suggested that isoantigenic modifications on the surface of cancer cells cause the appearance of blocking antibody, with broad cross-reactivity that might bind to the surface of responding and/or target cells.

INTRODUCTION

Impairment of cellular immunity in cancer patients has been documented by the decreased ability of such patients to develop delayed-type hypersensitivity reactions and to reject allogeneic grafts (20). The mediators of cellular immunity are thymus-derived lymphocytes, cells that display *in vitro* proliferative responses to PHA[2] and to mitomycin-treated allogeneic cells in MLC's. Thus, PHA and MLC reactivity can be used for *in vitro* evaluation of cellular immune responsiveness (12). Supporting evidence for decreased T-cell responsiveness in patients with cancer has been provided by studies on the PHA response (6, 9), but little is known about responsiveness in the MLC system. The MLC response is considered to be the *in vitro* counterpart of transplantation immunity, expressing the recognition phase of allograft rejection (19). The potential prognostic value of MLC determination in cancer derives from the axiom that, if lymphocytes from cancer patients display impaired responses to strong allogeneic transplantation antigens, they might not react efficiently against the comparatively weak autologous tumor-specific transplantation antigens. In addition, the MLC test seems suitable for investigation of serum-blocking factors as recently shown in transplantation studies (4, 18).

The present study evaluates the cellular immune responsiveness of patients with cancer and of control subjects by quantitating the lymphocyte response to PHA and to a battery of allogeneic cells. Investigations were designed to test the effect of cancer sera on the PHA and MLC responsiveness of autologous and of normal homologous lymphocytes.

MATERIALS AND METHODS

Sera and heparinized peripheral blood were obtained from patients with nonlymphoid malignant tumors who had never received radiation, chemotherapy, or blood transfusions. Healthy subjects in the same age range as the cancer patients were used as controls.

In Vitro **Lymphocyte Cultures.** A uniform technique for culturing, labeling, and harvesting the cells was used throughout the entire study. Lymphocyte suspensions were prepared from heparinized blood by the Ficoll-Isopaque gradient method (21) and were cultured in microtest plates as described by Hartzman *et al.* (13). All cells were washed 3 times and cultured in Roswell Park Memorial Institute Medium 1640 supplemented with glutamine, penicillin, and streptomycin. Cultures were set up by use of Hamilton

[1] This work was supported by Grant C-48408 from the State of New York, Department of Health, Kidney Disease Institute.

Received February 13, 1973; accepted June 25, 1973.

[2] The abbreviations used are: PHA, phytohemagglutinin; MLC, mixed-leukocyte culture.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/33/10/2373/2389901/cr0330102373.pdf by guest on 13 April 2024

syringes in a total volume of 0.2 ml of medium to which 0.05 ml of serum was added just before the start of incubation. Cells were incubated for 5 days at 37° in a humidified 4% CO_2 atmosphere, after which time 1 μCi of tritiated thymidine (specific gravity, 1.9 Ci/mM) was added to the cultures. Six hr later cultures were collected on glass fiber filters (Reeve-Angel 934AH) using an automatic multiple sample harvester. Label incorporation was measured by liquid scintillation counting in a Packard 3375 spectrometer. Differences in cpm between replicate cultures were less than 10%.

MLC Test. Responding lymphocytes were cultured in MLC with mitomycin-treated allogeneic lymphocytes ("stimulating cells") obtained from a selected panel of 10 healthy unrelated donors. The *HL-A* antigens covered by this selected panel were the following: *HL-A 1, 2, 3, 10, 11, W 28, AO 77* (of the 1st locus); and *HL-A 5, 7, 8, 12, W 10, W 15, W 27, AO 78* (of the 2nd locus). Quadruplicate cultures of responding cells were run against a standard stimulating cell mixture made up of equal amounts of cells from the 10 donors. Each culture contained 10^5 responding cells and 2×10^5 stimulating cells. The use of such stimulating cell mixtures in which 10 different *HL-A* phenotypes are represented is justified by our preliminary observation that this is a reproducible way of inducing maximum MLC response in any given population of responding cells. At the end of the incubation time viability was determined by trypan blue exclusion test using the 4th culture of the quadruplicates. Mean cpm of the remaining triplicate cultures was calculated for each of the responders, and the values were included in the statistical analysis of the results.

PHA Stimulation Test. Triplicate cultures of responding cells containing 2×10^5 lymphocytes/well were grown in the presence of purified PHA (Burroughs Wellcome Phytomitogen MR68) at a final concentration of 5.0 μg/ml.

Evaluation of Lymphocyte Responsiveness. Lymphocyte responsiveness was investigated in 60 patients with cancer and in 81 normal controls. Each subject's lymphocytes were tested simultaneously for PHA and MLC responsiveness in the presence of autologous serum and of homologous pooled sera from a healthy volunteer panel.

Screening of Inhibitory Serum Factors. The ability of sera from 60 cancer patients and from an equal number of normal controls to support *in vitro* lymphocyte blastogenesis was screened on a panel of responding lymphocytes obtained from 10 healthy staff members. All these subjects, except 3 of the cancer patients, belonged to the same population as the one that was tested for cellular reactivity. Responding lymphocytes from the panel members were challenged in parallel cultures with the standard stimulating cell mixture and with PHA. Mean cpm of triplicate cultures were calculated for each of the responders, and all of the 10 values obtained for 1 serum were included in the statistical analysis of the results.

Panel Cross-matches. Since cytotoxic antibodies have been shown to have an inhibitory effect on MLC, *HL-A* activity was investigated in all cancer sera by use of the microdroplet cytotoxicity method (23). Sera were screened on a random panel of 50 cells, including those used in the MLC reaction. Cells, complement, and positive and negative serum controls were included in each test. Staining of more than 25% of cells per well in more than 10% of lymphocyte samples was considered indicative of cytotoxic antibody activity.

RESULTS

Results obtained in cancer patients were grouped into 3 categories according to the extent of the tumoral growth at the time of testing: (*a*) tumors confined to the primary site; (*b*) tumors with regional spread; and (*c*) tumors with distant metastasis.

MLC and PHA Reactivity of Lymphocytes from Cancer Patients. Table 1 presents a comparison between lymphocyte reactivity of 60 cancer patients and of 81 normal controls. The MLC and PHA responsiveness of patients in any stage of malignant disease was significantly lower than that of controls ($p < 0.001$), regardless of whether the cells were cultured in autologous cancer or in pooled normal serum. MLC with autologous cancer serum additionally provided clear indications that the degree of deficiency was directly related to the extent of the disease. This is illustrated by the significantly lower reactivity of patients with metastatic cancer as compared to those in earlier stages. Such stage-dependent differences were obscured in the presence of normal serum, which restored the MLC reactivity of the lymphocytes from patients with regional and distant spread to more normal levels. A similar improvement, but to a much lesser degree, was observed in the PHA stimulation test, when normal instead of autologous cancer serum was used. This enhanced responsiveness cannot be attributed to an increased ability of the homologous pool of sera to support lymphocyte transformation since the blastogenic responses of lymphocytes from healthy controls stayed within the same range when tested in autologous or in homologous normal serum.

Effect of Serum from Cancer Patients on MLC and PHA Reactivity of Normal Panel Lymphocytes. Table 2 summarizes the results obtained by testing the effect of sera from 60 cancer and 60 normal subjects on MLC and PHA reactivity of lymphocytes from a constant panel of 10 healthy volunteers. For each group of sera, results were expressed as mean cpm in the total number of determinations. The statistical significance of differences between groups of cancer sera and normal, as well as the differences within the groups, was evaluated by the *t* test.

There was a significantly reduced rate of DNA synthesis in cultures grown in the presence of cancer sera as compared to normal sera. The inhibitory effect produced by cancer sera on the blastogenic response of normal lymphocytes increased with advanced disease, as shown by the significantly lower rate of thymidine incorporation in cultures containing sera from patients with metastatic cancer than in those with sera from earlier stages. The amplitude of the differences among the groups was higher in MLC's than in PHA-stimulated cultures.

HL-A Antibody Activity in Cancer Sera. Table 3 represents the results obtained by examining the lymphocyte

Downloaded from http://aacrjournals.org/cancerres/article-pdf/33/10/2373/2389901/cr0331002373.pdf by guest on 13 April 2024

Table 1

MLC and PHA responsiveness of lymphocytes from cancer and normal subjects

Responsiveness is expressed as tritiated thymidine incorporation in triplicate cultures of lymphocytes from each subject included in the group. All cultures were harvested after 5 days and radioactivity was determined by liquid scintillation counting.

Group of subjects	No. of cases	Incorporation (cpm)	
		Autologous serum	Normal pool serum
		MLC	
Normal control	81	26,400[a] ± 200[b]	25,521[a] ± 162
Cancer patients			
Primary tumors	16	14,894 ± 792	14,512 ± 816
Regional spread	23	15,816 ± 420	21,459 ± 985
Distant spread	21	8,968 ± 459	16,673 ± 690
		PHA	
Normal control	81	23,250[a] ± 210	22,433[a] ± 180
Cancer patients			
Primary tumors	16	17,501 ± 124	18,475 ± 740
Regional spread	23	13,345 ± 540	15,285 ± 650
Distant spread	21	10,516 ± 480	12,857 ± 460

[a] The difference between the responsiveness of lymphocytes from the group of normal subjects and all groups of cancer patients is significant ($p < 0.001$).

[b] Mean ± S.E.

Table 2

Effect of cancer sera on MLC and PHA responsiveness of lymphocytes from normal subjects

The blastogenic activity of lymphocytes was evaluated by determination of tritiated thymidine incorporation.

Source of sera	No. of sera	No. of determinations[a]	Incorporation (cpm)[b]	Comparison to		
				Normal	Cancer	
					Primary	Regional
			MLC			
Normal	60	600	27,249 ± 24[c]			
Primary tumors	18	180	23,411 ± 89	<0.005		
Regional spread	20	200	20,786 ± 68	<0.001	<0.05	
Distant spread	22	220	12,983 ± 35	<0.001	<0.001	<0.001
			PHA			
Normal	60	600	22,433 ± 13			
Primary tumors	18	180	18,093 ± 31	<0.001		
Regional spread	20	200	19,822 ± 49	<0.001		
Distant spread	22	220	16,102 ± 35	<0.001	<0.05	<0.001

[a] The inhibitory effect of each serum was tested on a constant panel of responding lymphocytes from 10 healthy volunteers.

[b] All 10 values obtained for 1 serum were included in the calculation of mean cpm for each group.

[c] Mean ± S.E.

Table 3

Cytotoxic activity of cancer sera

Serum from each cancer patient was tested on a random panel of lymphocytes from 50 healthy subjects for antibody screening.

	Sera from cancer patients with			
	Primary tumors	Regional spread	Distant metastasis	Total sera
Total sera tested	18	20	22	60
No. of positive sera from				
Males	1	3	6	10
Females	5	4	4	13

cytotoxicity of cancer sera. Twenty-three out of 60 sera reacted with more than 10% of the panel cells, showing multispecific anti-*HL-A* activity. Reactions were generally weak, as manifested by 25 to 50% killed lymphocytes in nondiluted serum. Transfusions were excluded as a possible cause for the appearance of cytotoxic antibodies (since only nontransfused patients were included in this study) but pregnancy antibodies might have been involved in females. Sera with antibody activity consistently induced inhibition of lymphoblastic transformation. The blocking effect was, however, not dependent on the cytotoxic activity, as indicated by the fact that (*a*) noncytotoxic sera produced a similar degree of inhibition, and (*b*) the presence of cytotoxic antibody did not affect the viability of the cultures.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/33/10/2373/2389901/cr0330102373.pdf by guest on 13 April 2024

DISCUSSION

Our present investigations demonstrate that lymphocytes from cancer patients have a decreased ability to respond to *in vitro* stimulation with allogeneic cells and with PHA. An inverse correlation has been found between the blastogenic response of lymphocytes from cancer patients and the extent of the disease. These observations might indicate that circulating T-lymphocytes, for which PHA and MLC responsiveness are reliable functional markers, are progressively depleted during advancement of cancer or that they are replaced by a population bearing an intrinsic defect.

However, our finding that the MLC responsiveness of lymphocytes from patients with advanced disease can be restored to higher levels by growing the cultures in normal instead of autologous cancer serum shows that a humoral factor is at least partially responsible for the impairment of cellular reactivity. Sera from cancer patients displayed a stage-related inhibitory effect, altering the MLC responsiveness of lymphocytes from normal panel members. The presence of factors in cancer sera that are capable of blocking the blastogenic function of both autologous and normal homologous lymphocytes was further confirmed by use of the PHA stimulation test. However, as previously noted (1, 11, 25), the existence of blocking factors cannot be firmly ascertained by the PHA stimulation technique probably because the degree of inhibition is relatively low even when sera from advanced cases are used.

Impairment of MLC reactivity of autologous and homologous lymphocytes by cancer serum is reminiscent of the MLC-inhibitory effect produced by sera from multiparous women and transplant recipients (4, 18). This last phenomenon was ascribed to modulation of cell-mediated immunity by blocking antibodies with a broad cross-reactivity for *HL-A* antigens and/or for the closely linked immune receptors on the surface of T-lymphocytes (2, 4). By analogy, one can postulate that the MLC-blocking activity of cancer sera is also due to multispecific anti-*HL-A* antibodies which might have developed in response to new isoantigens arising on the surface of malignant cells.

Direct evidence is lacking, but as shown in our and other authors' studies, a relatively high incidence of anti-*HL-A* activity was found in cancer sera, while new isoantigens that are absent on the patients' normal cells were detected in some human tumors (3, 22).

If serum-blocking factors display *in vivo* the same lack of isoantigenic specificity as demonstrated *in vitro* by their ability to inhibit the reactivity of homologous lymphocytes, it might be conceivable that they could act dually on target cells, leading to efferent enhancement of the tumor, and on T-lymphocytes, leading to central impairment of cellular immune responsiveness. Such a mechanism offers a tempting explanation for the anergy of cancer patients to tumoral growth as well as to standard bacterial, fungal, and viral infections (10).

Recent studies show that lymphocytes from patients with progressively growing cancer selectively destroy cells derived from their own tumor or from other tumors of the same histological type (15). Since normal cells from the same patients were not destroyed by sensitized lymphocytes, it has been inferred that cell-mediated immunity is directed against antigens that are qualitatively or quantitatively different from normal isoantigens (14, 15). Sera from such patients contain antibodies that block cell-mediated tumor immunity showing the same group-specific cross-reactivity as the lymphocytotoxicity itself. The broad inhibitory activity of cancer sera in the lymphocytotoxicity system is similar to that displayed toward lymphocytes from unrelated subjects in the lymphocyte blastogenesis system and might be due to the same factor. The possibility exists that inhibition is produced in both cases by a blocking antibody with wide cross-reactivity for histocompatibility-linked T-cell receptors.

Alternatively, since a clear distinction exists between factors blocking tumor immunity *in vitro* and serum lymphocyte depression which effects antigenically unrelated responses, it may turn out that the depressor is not an immunoglobulin but some other class of regulator.

Serum-inhibitory factors have also been demonstrated in patients with a variety of (destructive) neurological diseases, tuberculosis, histoplasmosis, secondary syphilis, chronic candidiasis, leprosy, hepatitis, uremia, etc. (7, 8, 16, 17). The nature and significance of the suppressive serum factors has not yet been established and the possibility exists that they might be qualitatively different in various diseases. The common traits of such factors, however, are their ability to inhibit the responsiveness of autologous as well as homologous lymphocytes and their occurrence in elevated titer in diseases that are associated with organ destruction and lymphocyte sensitization to a variety of antigens (7, 8). This had led to the hypothesis that the suppressor element might indeed be the feedback factor (8) that controls the lymphocyte reactivity to self and non-self antigens (5, 24).

The conclusion cannot be escaped that, in view of the possible therapeutic manipulation of the level of serum-inhibitory factors in a variety of clinical conditions such as cancer, autoimmune diseases, and transplantation, their isolation and characterization is a chief challenge which now lies ahead of us.

ACKNOWLEDGMENTS

The authors are grateful to Professor G. T. V. Nossal for his invaluable suggestions and advice. Our thanks are extended to Fahim Elnady and Pilarita Almojera for their excellent technical assistance and to Donna Angioletti and Sonja Fenner for help in preparing the manuscript.

REFERENCES

1. Al-Saraf, M., and Vaitkevicius, V. K. Effect of Homologous Plasma on Lymphocyte Transformation in Cancer Patients and Controls. Lancet, *2:* 314-315, 1971.
2. Benacerraf, B., and McDivitt, H. O. Histocompatibility-linked Immune Response Genes. Science, *175:* 273, 1972.
3. Biedler, J., Helson, L., and Suciu-Foca, N. Expression of *HL-A* Antigens on Human Neuroblastoma Cells in Continuous Culture. Ab-

Downloaded from http://aacrjournals.org/cancerres/article-pdf/33/10/2373/2389901/cr0330102373.pdf by guest on 13 April 2024

stracts of the 24th Annual Meeting of the Tissue Culture Association, Inc., in press.

4. Ceppellini, R., Bonnard, G. D. Coppo, F., Miggiano, V. C., Pospisil, M., Curtoni, E. S., and Pellegrino, M. Mixed Leukocyte Cultures and *HL-A* Antigens: I. Reactivity of Young Fetuses, Newborns and Mothers at Delivery. II. Inhibition by Anti *HL-A* Sera. Transplant. Proc., *3:* 58-70, 1971.
5. Cohen, I. R. Cell-mediated Autoimmunity: Antigen Reactive Lymphocytes Recruit Specific Effector Lymphocytes. Nature New Biol., *242:* 60-61, 1972.
6. Ducos, J., Migueres, J., and Colombies, P. Lymphocyte Response to PHA in Patients with Lung Cancer. Lancet, *1:* 111-112, 1970.
7. Field, E. J., and Caspary, E. A. Inhibition of Lymphocyte Response by Serum. Lancet *2:* 95-96, 1971.
8. Field, E. J., and Caspary, E. A. Lymphocyte Response Depressive Factor in Multiple Sclerosis. Brit. Med. J., *4:* 529-532, 1971.
9. Garrioch, D. B., Good, R. A., and Gatti, R. A. Lymphocyte Response to PHA in Patients with Non-Lymphoid Tumors. Lancet, *1:* 618, 1970.
10. Gold, P. Immunologic Technology in the Diagnosis of Cancer. Bull. Cancer, *9:* 1-3, 1971.
11. Golob, E. K., Israsena, T., Quatrale, A. C., and Becker, K. L. Effect of Serum from Cancer Patients on Homologous Lymphocyte Cultures. Cancer, *23:* 306-308, 1969.
12. Good, R. A. Structure-Function Relations in the Lymphoid System. Clin. Immunol., *1:* 1-28, 1972.
13. Hartzman, R. J., Segall, M., and Bach, F. H. Histocompatibility Matching. Miniaturization of The Mixed Leukocyte Culture Test. Transplantation, *11:* 268-273, 1971.
14. Haywood, G. R., and McKhann, C. F. Antigenic Specificities on Murine Sarcoma Cells. Reciprocal Relationships between Normal Transplantation Antigens (*H2*) and Tumor Specific Immunogenicity. J. Exptl. Med., *133:* 1171-1187, 1971.
15. Hellström, I., and Hellström, K. E. Some Aspects of the Immune Defense against Cancer. II. *In Vitro* Studies on Human Tumors. Cancer, *28:* 1269-1271, 1971.
16. Huber, D., Pastner, P., Dittrich, P., and Braunsteiner, H. *In Vitro* Reactivity of Human Lymphocytes in Uremia—A Comparison with the Impairment of Delayed Hypersensitivity. Clin. Exptl. Immunol., *5:* 75-82, 1969.
17. Newberry, W. M., Shorey, J. W., Sanford, J. P., and Combes, B. Depression of Lymphocyte Reactivity to Phytohemagglutinin by Serum from Patients with Liver Disease. Cellular Immunol., *6:* 87-97, 1973.
18. Revillard, J. P., Robert, M., DuPont, E., Betuel, H., Rifle, G., and Traeger, J. Inhibition of Mixed Lymphocyte Culture by Alloantibodies in Renal Transplantation and in Pregnancy. Transplant. Proc., *5:* 331-335, 1973.
19. Sorensen, S. F. The Mixed Lymphocyte Culture Interaction. Acta Pathol. Microbiol. Scand. B, Suppl. 230, 1972.
20. Southam, C. M. The Immunologic Status of Patients with Nonlymphomatous Cancer. Cancer Res., *28:* 1433-1440, 1968.
21. Suciu-Foca, N., Buda, J., Thiem, S., and Suciu, T. Serial Determination of Blast Cell Count during Allograft Rejection in Rats. Transplantation, *14:* 711-715, 1972.
22. Takasugi, M., and Terasaki, P. I. Detection of *HL-A* and Other Cell-Surface Antigens on Cultured Cells by a Cytotoxic Plating Inhibition Test. J. Natl. Cancer Inst., *49:* 1229-1237, 1972.
23. Terasaki, P., McCurdy, B., and McClelland, J. Microdroplet Lymphocyte Cytotoxicity Test. *In:* J. G. Ray, R. C. Scott, D. B. Hare, C. E. Harris, and D. E. Kayhoe (eds.), Manual of Tissue Typing Techniques, pp. 50-55. Bethesda, Md.: National Institute of Health, 1972.
24. Wekerle, H., Cohen, I. R., and Feldman, M. Lymphocyte Receptors for Autoantigens, Autologous Serum Inhibits Self-recognition. Nature New Biol., *241:* 25-26, 1973.
25. Whittaker, M. G., Clark, C. G., and Rees, K. Reduced Lymphocyte Transformation in Breast Cancer. Lancet, *1:* 892-893, 1971.

Downloaded from http://aacrjournals.org/cancerres/article-pdf/33/10/2373/2389901/cr0330102373.pdf by guest on 13 April 2024