PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 157, 511-516 (1978)

Effect of the Prolonged Immunization with Tumor-Unrelated Antigen on the Tumor Growth[1] (40087)

STANISLAW P. TARGOWSKI, BORIS ALBINI, AND FELIX MILGROM

Department of Microbiology, State University of New York at Buffalo, School of Medicine, Buffalo, New York 14214

Sera from tumor-bearing men and animals contain immune complexes (1, 2). It was shown that immune complexes in the sera from tumor-bearing animals are composed of the tumor antigen and its corresponding antibody (2). These sera may block cytotoxic activity of lymphocytes *in vitro* (for references, see 3) and enhance tumor growth (4). One could imagine that immune complexes from sera of the tumor bearers may specifically bind to lymphocytes or nonspecifically attach to Fc receptors of killer lymphocytes and inactivate them. These complexes may also attach to tumor cells in the same manner and mask tumor specific transplantation antigen. This raises the question whether tumor-unrelated immune complexes can attach to Fc receptors of the killer lymphocytes or tumor cells and inhibit anti-tumor response of the host. If this were the case, enhancement of tumor growth in the host with high level of the circulating immune complexes should be expected.

Formation of immune complexes in circulation and their deposition in the kidneys have been observed in rabbits (5) and mice (6) injected with antigen for a period of several months. In the present study, tumor growth was investigated in mice immunized for 4–5 months with tumor-unrelated antigens.

Materials and methods. Animals. C3H/HeHa mice, 12 weeks old, from West Seneca Laboratories, West Seneca, N.Y., were used in this study. The immunized and control animals were without any clinical signs of infection or sickness during the experimental period. Death of 30% of the immunized mice was due to the anaphylactic shock.

Tumor tissue. Methylcholanthrene-induced sarcoma, MCSa-22 was originally produced in this laboratory by subcutaneous injection of the carcinogen into a C3H/HeHa mouse. This tumor was maintained by transplantation in the strain of origin. Tumor tissue collected within 2–3 weeks after transplantation into normal mice was used in hemadsorption tests and/or for preparation of the tumor cell suspensions. Neoplastic tissue was minced, trypsinized and washed. Then suspensions were prepared that contained 10^5, 10^4, 10^3 or 10^2 cells in 0.2 ml of TC-199 medium supplemented with 0.02 *M* L-glutamine, 100 U of penicillin and 100 mg streptomycin/ml. These tumor cells were injected subcutaneously into experimental (immunized) and control (unimmunized) mice. Cryostat sections of tumors collected from immunized and control mice were tested for hemadsorption (see below).

Immunization and immune sera. Mice from one group were immunized daily for 4 to 5 months with bovine serum albumin (BSA) (Miles Laboratories, Inc., Elkhart, IN). The antigen was injected intravenously (iv) into the caudal tail vein at the dosage of 0.2 mg in 0.2 ml of phosphate buffered saline (PBS). Mice from another group were not immunized and served as a control. Mice from both groups were trial bled at the end of the third month of the immunization before injection with tumor cells and were terminally bled in the 4th and 5th month of the experimental period.

In another experiment, mice were injected intraperitoneally (ip) with ovalbumin (OA) (Sigma Chemical Company, St. Louis, MO). Here, again, daily injections of 0.2 mg of antigen in 0.2 ml of PBS were administered. The immune mice and corresponding control mice were bled out at the end of the 4th month of the experimental period.

All sera were stored at −70° and thawed quickly before testing. These sera were tested for circulating immune complexes and for inhibition of hemadsorption (see below).

Hemadsorption and inhibition of hemad-

[1] Supported by Grant No. IM-24B from the American Cancer Society.

0037-9727/78/1573-0511$01.00/0

sorption. Hemadsorption by cryostatcut tissue sections was performed as previously described (7). Briefly, 10 μm thick tissue sections were placed on coverslips and dried at room temperature. Wells of microculture slides were filled with a suspension of sheep erythrocytes sensitized by a selected subagglutinating dilution of a rabbit anti-sheep erythrocyte serum. The wells were sealed with the coverslips in such a way that the tissue section was submerged in the suspension of sensitized erythrocytes. The preparations were incubated coverslip down for 1 hr at room temperature. Then they were turned coverslip up and left in this position at room temperature to allow unattached erythrocytes to fall down from the tissue. The sections were examined microscopically at low magnification. Adherence of erythrocytes to the sections was recorded as a positive hemadsorption test. If a tissue section was completely covered by erythrocytes, the test was graded as 4+. A reaction was considered 2+ if approximately half the tissue was covered by erythrocytes. Sparsely attached erythrocytes on the tissues were considered as a 1+ reaction. Absence of erythrocytes from the tissue was recorded as a negative result.

Inhibition studies were performed by incubating tissue sections with various sera for 20 min in a moist chamber at room temperature (8). Subsequently, the tissue sections were washed three times in PBS and the hemadsorption test was performed as described above. The lowest concentration of rabbit anti-sheep erythrocyte serum used for sensitization of sheep erythrocytes which showed 4+ hemadsorption was determined for each tumor specimen and employed in the inhibition studies.

Detection of immune complexes. Immune complexes in circulation were detected by using the Raji cell test (9) in a modification recently described (10). Briefly, a suspension of Raji cells (kindly supplied by Dr. T. Flanagan of the Department of Microbiology, SUNY at Buffalo, Buffalo, N.Y.) were harvested 72 hr after transfer. Fifty μl of Raji cells in RPMI-1640 medium (Associated Biomedic Systems, Inc., Buffalo, N.Y.) at a concentration of 5×10^6 cells/ml were incubated at 4° for 30 min with 50 μl of the tested serum or one of its dilutions. Subsequently, cells were washed with RPMI medium and stained at 4° for 30 min with 25 μl of fluorescein isothiocyanate (FITC)-labeled rabbit anti-murine IgG serum, with 25 μl of FITC-labeled rabbit anti-BSA serum, or with 25 μl of FITC-labeled rabbit anti-OA serum. Cells were then washed twice in RPMI medium and twice in PBS. One drop of the suspension was placed on a microscope slide and dried under a fan. Raji cells, incubated in the medium and then stained with FITC-conjugated sera, served as a control. Immune complexes in the cryostat-cut sections of the kidney were detected by staining with the same FITC-conjugated rabbit antisera.

Experimental design. At the time when immune complexes were detected in the kidneys from randomly selected immunized animals (approximately at 3 months of the immunization), all experimental and corresponding control mice were divided into groups and injected with 10^5, 10^4, 10^3 or 10^2 tumor cells. Cells from the 22nd passage of the syngenic tumor were injected into OA-immunized and corresponding control mice, whereas BSA immunized mice and corresponding control mice were injected with the cells from the 33rd passage of the same transplantable tumor. Subsequently, growth of the tumor was inspected daily. At the end of the experiment, all animals were bled out and the tumor was excised and weighed.

Statistical analysis. Data were analyzed by student's *t* test.

Results. Rate of tumor growth. At the time when deposits of immune complexes in the kidneys and circulating immune complexes in the sera of BSA-immunized mice could be demonstrated, the mice were divided into three groups and injected with 10^4, 10^3 and 10^2 tumor cells, respectively. Three groups of control mice were injected with similar numbers of the tumor cells. Figure 1 shows the results of this experiment. In all immunized and control mice injected with 10^4 and 10^3 tumor cells, tumors developed in 8–17 days after injection. All immunized mice injected with 10^2 tumor cells developed tumors at the same time. However, only three of ten control mice injected with 10^2 tumor cells developed tumors at that time. Three additional mice of this group developed tumors within 43 days.

Figure 2 shows the effect of prolonged

immunization with OA on the rate of tumor growth. When immune complexes appeared in the kidneys of OA-injected mice, immunized and control mice were divided into three groups and injected with 10^5, 10^4 and 10^3 tumor cells. Tumors were detectable on the 12th day after injection in all mice except the control mice injected with 10^3 tumor cells. In this group, tumor developed only in six of 12 mice and the remainder did not change during 21 days of observation.

Tumor weight. In the experiments with BSA immunization, the tumors from mice injected with 10^4, 10^3 and 10^2 cells were excised and individually weighed on the 28th, 35th and 43rd day after injection, respectively. Table I demonstrates the effect of long-term immunization with BSA on the weight of tumor mass. In all groups, tumors from immunized mice weighed significantly more than tumors from control animals.

Tumors from OA-immunized mice and corresponding control mice were excised and weighed on the 21st day after injection with 10^5 and 10^3 tumor cells. Weight of the tumors from immunized mice injected with 10^5 tumor cells was significantly greater than that of control mice injected with the same number of tumor cells. Tumor weight did not significantly differ between immunized and control mice injected with 10^3 of the tumor

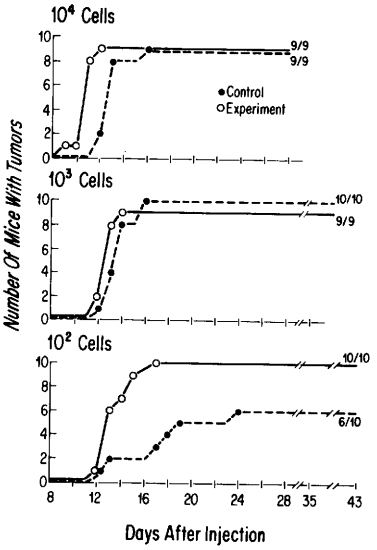

FIG. 1. Tumor growth in BSA-immunized and control mice injected with 10^4, 10^3 and 10^2 syngenic tumor cells.

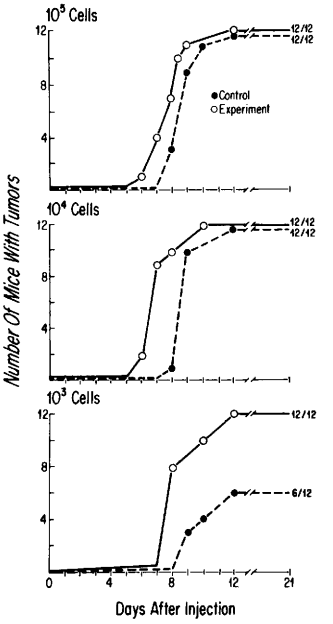

FIG. 2. Tumor growth in OA-immunized and control mice injected with 10^5, 10^4 and 10^3 syngenic tumor cells.

TABLE I. EFFECT OF PROLONGED IMMUNIZATION OF MICE WITH BSA OR OA ON THE WEIGHT OF THE TUMOR.

	Weight of tumors (mg) from mice injected with tumor cells			
Mice	10^5	10^4	10^3	10^2
Immunized[a] with BSA	ND[b]	1326 ± 336[c e]	1438 ± 209[e]	1831 ± 354[e]
Control	ND	470 ± 72	433 ± 86	416 ± 140
Immunized[d] with OA	1235 ± 266[e]	ND	385 ± 108	ND
Control	448 ± 90	ND	272 ± 102	ND

[a] Tumors from BSA-immunized and control mice injected with 10^4, 10^3, and 10^2 tumor cells were excised and weighed on the 28th, 35th and 43rd day after injection, respectively.

[b] Not done.

[c] Mean ± SD.

[d] Tumors from OA-immunized and control mice injected with 10^5 and 10^3 tumor cells were excised and weighed on the 21st day after injection.

[e] Denotes statistically different from control at $P<0.05$.

cells (Table I).

Hemadsorption and inhibition of hemadsorption. Cryostat sections of tumors from mice immunized with BSA or OA and control mice did not show any difference in the adsorption of the sensitized sheep erythrocytes. Sera from tumor-bearing mice immunized with either BSA or OA markedly inhibited hemadsorption, whereas, sera from tumor-bearing non-immunized mice and from normal mice did not inhibit hemadsorption. Table II presents results of such inhibition experiments in which hemadsorption by tumor tissue and by normal spleen tissue was examined.

Immune complexes were deposited in kidneys of the mice after 2 to 3 months of the injections with BSA or OA. These complexes were stained with FITC-labeled rabbit anti-murine IgG. In some experiments they were stained with FITC-labeled rabbit anti-BSA or rabbit anti-OA, respectively. Before tumor cells were injected into the mice, the immune complexes were demonstrated in all of the kidneys taken from five randomly selected mice of the group of the animals immunized with BSA and five mice immunized with OA. In the kidneys collected when the tumors were harvested from the immunized mice with either BSA or OA, immune complexes were detected in 80% by staining with anti-BSA serum or anti-OA serum, respectively, and in 100% by staining with anti-murine IgG serum. Immune complexes in the kidneys taken from control (unimmunized) mice injected with tumor cells were not detected by using anti-BSA serum or anti-OA serum. However, they were detected in 40% of mice by staining with anti-murine IgG serum. A similar percentage of the positive staining was also noticed in the kidneys from normal mice. Deposition of IgG has previously been described in kidneys of aging mice (11).

TABLE II. ADSORPTION OF SENSITIZED SHEEP ERYTHROCYTES BY C3H TUMOR AND SPLEEN TISSUES. INHIBITION BY SERA FROM NORMAL MICE, TUMOR-BEARING UNIMMUNIZED MICE AND TUMOR-BEARING IMMUNIZED MICE.

Hemadsorption by:	Serum dilution 1 to:	Serum from normal mouse	Sera from tumor-bearing mice	
			Unimmunized	Immunized
Tumor tissue	10	4+	3+	—
	20	4+	4+	—
	40	4+	4+	2+
Spleen tissue	10	4+	3+	—
	20	4+	4+	1+
	40	4+	4+	1+

The same FITC-labeled reagents were used to demonstrate circulating immune complexes in sera of immunized mice by means of the Raji cell assay. Using anti-BSA serum, the average titer of the circulating immune complexes was 1:8 ranging from 1:2 to 1:32 in the individual sera from trial bleeding of BSA-immunized mice. This titer decreased to 1:2 ranging from 1:1 to 1:4 in the sera from terminal bleeding of the BSA-immunized mice. In the sera from terminal bleeding of OA-immunized mice, the circulating complexes were detected in the average titer of 1:4 ranging from 1:2 to 1:16. OA anti-OA complexes were not tested in the sera from trial bleeding of OA-immunized mice. Immune complexes composed of BSA or OA and their corresponding antibodies were not detectable in the sera from control mice injected with tumor cells or from normal mice.

Using FITC-labeled rabbit anti-murine IgG serum in Raji cell assay with sera from trial bleeding of BSA-immunized mice, circulating immune complexes were in the average titer of 1:32 ranging from 1:8 to 1:64. A similar titer of circulating immune complexes was observed in the sera from the terminal bleeding of BSA- or OA-immunized tumor-bearing mice and control mice injected with tumor cells. Interestingly, the circulating immune complexes were also detected in the sera from normal mice at the average titer of 1:8 ranging from 1:4 to 1:32. The increased amount of circulating immune complexes in control mice injected with tumor cells was possibly due to tumor growth.

Discussion. The rate of tumor growth in mice immunized with BSA and injected with 10^2 tumor cells and in mice immunized with OA and injected with 10^3 tumor cells was markedly greater than that in control non-immunized mice (Figs. 1 and 2). Tumor weight was also significantly greater in mice immunized with BSA and OA than nonimmunized mice (Table I). Similar enhancement of tumor growth was noted in immunologically impaired mice injected with a mixture of the tumor cells and sensitized lymphocytes (12, 13). Jeejeebhoy (14) has reported that a certain population of the sen-

sitized lymphocytes is responsible for enhancement and another for inhibition of tumor growth. Lymphocytes collected on the 5th day after injection of tumor cells into mice enhanced growth in the tumor *in vitro*, whereas lymphocytes collected by day 12 inhibited formation of the neoplastic cell colonies. Recent observations suggested that immunostimulation of tumor growth *in vitro* is mediated by T and B cells that are distinct from cytotoxic effector cells (15).

The data of this study demonstrated that 50% of control mice injected with 10^3 tumor cells developed tumors in one experiment (Fig. 2), whereas in another experiment performed 5 months later, the tumors developed in 100% of the control mice injected with the same dose of the same tumor (Fig. 1). This variation could be due to the changes in the virulence of this tumor with the progressing number of the passages. Another possibility is that cells from the tumor used in one experiment contained relatively more macrophages than the cells from the tumor employed in another experiment. The number of macrophages in the inoculum may obviously have influence on the tumor growth as observed by Wood and Gillespie (16).

In our study, mice were immunized with tumor-unrelated antigens for 4–5 months. In 2–3 months, deposition of immune complexes composed of BSA or OA and corresponding antibodies in kidneys was observed. The immune complexes were detected in sera of these mice by Raji cell assay and by inhibition of hemadsorption test. Other investigators observed deposition of immune complexes in kidneys of F1 hybrid mice with graft-versus-host reaction resulting from transplantation of parental lymphoid cells (17). It was suggested that antigens of the hybrid formed immune complexes with their corresponding antibodies produced by the parental cells and that these complexes were deposited in the kidneys (18). Furthermore, continuous antigenic stimulation of the immune system in animals with graft-versus-host reaction lead to an increased incidence of neoplasia (19). It is quite possible that in our study, daily injection of an antigen continuously stimulated lymphocytes which, in turn, could contribute to the enhancement of the tumor growth.

Daily injection of antigens could also result in continuous formation of circulating immune complexes which could attach to Fc receptors of the cytotoxic lymphocytes and inactivate these cells (20, 21). A recent study indicated that most mouse cytotoxic lymphocytes possess Fc receptors (22). In addition, circulating immune complexes could attach to the phagocytic cells and decrease their ability to penetrate into the site of tumor cell injection (23). A decreased number of phagocytic cells at the tumor site might have affected tumorcidal activity, attraction of lymphoid cells into tumor site and the interaction between macrophages and lymphocytes which, in turn, might have diminished immune response to the tumor antigen.

Alternatively, circulating immune complexes could also attach to the tumor cells and mask tumor specific transplantation antigens; therefore, the immune system of the host could not recognize them. This suggestion is supported by the observation that immune complexes can attach to the Fc receptors of tumor tissue (24) or tumor cells (25).

Summary. The effect of prolonged immunization of mice with bovine serum albumin (BSA) or ovalbumin (OA) on the growth of the transplanted, methylcholanthrene-induced fibrosarcoma was studied. When immune complexes appeared in the kidneys and in the circulation, mice were injected subcutaneously with various numbers of syngeneic tumor cells.

Tumor developed within 17 days in all mice immunized with BSA and injected with 10^4, 10^3 and 10^2 tumor cells and control mice injected with 10^4 and 10^3, whereas only 3 of 10 control mice injected with 10^2 tumor cells developed tumors at that time. All mice immunized with OA and injected with 10^5, 10^4 and 10^3 tumor cells and control mice injected with 10^5 and 10^4 developed tumor within 12 days after injection of the tumor cells, whereas in the control group injected with 10^3 tumor cells, only 6 of 12 mice did so. Weight of the tumors from the mice immunized with BSA or with OA was significantly greater than that of tumors from control mice.

The authors are grateful to Mrs. S. Sieczkarski for her excellent technical assistance.

1. Theofilopoulos, A. N., Andrews, B. S. Urist, M. M.,

Morton, D. L., and Dixon, F. J., J. Immunol. **119,** 657 (1977).
2. Tamerius, J., Nepon, J., Hellström, I., and Hellström, K. E., J. Immunol. **116,** 724 (1976).
3. Hellström, K. E., and Hellström, I., Advan. Immunol. **18,** 209 (1974).
4. Bansal, S. L., Bansal, B. R., and Boland, J. P., Curr. Top. Microbiol. Immunol. **75,** 45 (1976).
5. Unanue, E., and Dixon, F. J., Advan. Immunol. **6,** 1 (1967).
6. Okumura, K., Acta Pathol. Jap. **23,** 695 (1973).
7. Milgrom, F., Humphrey, L. J., Tönder, O., Yasuda, J., and Witebsky, E., Int. Arch. Allergy **33,** 478 (1968).
8. Targowski, S. P., Abeyounis, C. J., and Milgrom, F., Proc. Soc. Exp. Biol. Med. **154,** 365 (1977).
9. Theofilopoulos, A. N., Wilson, C. B., Bokish, V. A., and Dixon, F. J., J. Exp. Med. **140,** 1230 (1974).
10. Albini, B., Ossi, E., and Andres, G., Lab. Invest. **37,** 64 (1977).
11. Gude, W. D., and Upton, A. C., Amer. J. Pathol. **40,** 699 (1962).
12. Prehn, R. T., Science **176,** 170 (1972).
13. Carnard, C., Ilfeld, D., and Levo, Y., Int. J. Cancer **14,** 168 (1974).
14. Jeejeebhoy, H. F., Int. J. Cancer **13,** 665 (1974).
15. Norbury, K. C., Cancer Res. **37,** 1408 (1977).
16. Wood, G. W., and Gillespie, G. Y., Int. J. Cancer **16,** 1022 (1975).
17. Lewis, R. M., Armstrong, M. Y. K., Andre-Schwartz, J., Muftuoglu, A., Beldotti, L., and Schwartz, R. S., J. Exp. Med. **128,** 653 (1968).
18. Kano, K., Beldotti, L., Milgrom, F., and Schwartz, R. S., J. Immunol. **112,** 410 (1974).
19. Armstrong, M. Y. K., Gleichmann, E., Gleichmann, H., Beldotti, L., Andre-Schwartz, J., and Schwartz, R. S., J. Exp. Med. **132,** 417 (1970).
20. Gorczynski, R. M., Kilburn, D. G., Knight, R. A., Norburh, C., Parker, D. C., and Smith, J. B., Nature (London) **254,** 141 (1975).
21. Kilburn, D. G., Fairhurst, M., Levy, J. G., and Whitney, R. B., J. Immunol. **117,** 1612 (1976).
22. Herberman, R. B., Bartram, S., Haskill, J. S., Nunn, M., Holden, H. T., and West, W. H., J. Immunol. **119,** 322 (1977).
23. Eccles, S. A., Bandlow, G., and Alexander, P., Br. J. Cancer **34,** 20 (1976).
24. Targowski, S. P., Abeyounis, C. J., and Milgrom, F., Int. Arch. Allergy **54,** 262 (1977).
25. Braslawsky, G. R., Serban, D., and Witz, I. P., Europ. J. Immunol. **6,** 579 (1976).

Received September 19, 1977. P.S.E.B.M. 1978, Vol. 157.