[CANCER RESEARCH 35, 2646-2650, October 1975]

Tumor Immunoprophylaxis in Mice Using Glutaraldehyde-treated Syngeneic Tumor Cells[1]

P. Frost[2, 3] and C. J. Sanderson

Division of Surgical Sciences, Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ, England

SUMMARY

The ability of glutaraldehyde-treated tumor cells to induce protection against subsequent challenge has been studied in a syngeneic system. Two tumors have been tested in BALB/c mice. The first was a methylcholanthrene-induced tumor that has been maintained in serial passage for over a decade. The second was a spontaneous mammary adenoacanthoma that was tested at the third passage. The protection was found to consist of two components: (*a*) a specific immunological component; and (*b*) a nonspecific component observed when the immunizing and challenge dose were both given i.p. This nonspecificity, while possibly an element in tumor protective mechanisms, may confuse careful analytical studies of the immunogenic potential of tumor antigens.

INTRODUCTION

There is as yet very little good evidence that specific active immunotherapy can significantly alter the growth pattern of preexisting tumors. There are 2 possible reasons for this: the host may already be undergoing a maximal immune response made ineffective by such factors as the tumor growth rate and antigen shedding (24); the natural presentation of tumor antigens to the host may be inadequate for the initiation of an effective immune response, a possibility that has led to the study of tumor antigenicity utilizing a variety of prophylactic models. A basic premise of these studies was that once improved conditions for tumor antigen presentation were achieved a more careful study of the host response could be undertaken.

In a preliminary report (23) we described the efficacy of glutaraldehyde-treated Meth A[4] tumor cells in protecting against subsequent challenge with viable tumor cells. It was demonstrated that, 14 days after i.p. injection of 5×10^7 glutaraldehyde-treated tumor cells, mice were fully protected against an i.p. challenge of 10^5 tumor cells. In this paper we extend these studies and make use of a tumor that arose spontaneously in our colony. The new tumor has enabled us to study this phenomenon at the 3rd passage.

Attempts to modify tumor tissue so as to increase its immunogenicity while attenuating its virulence have included a wide variety of physical and chemical treatments. Irradiation of tumor cells (10, 21) was one of the earliest means of achieving immune protection against methylcholanthrene-induced tumors. Other physical treatments such as heating, lyophilization (20), or sonication (16) have been shown to be ineffective. The coupling of new antigens to tumor cells has, however, been shown to increase immunogenicity (14), as measured by *in vitro* cell-mediated cytotoxicity.

Another means of inducing tumor immunity has involved chemical modification of tumor cells. Agents such as iodoacetate and iodoacetamide, when used to treat tumor cells, have produced protection against viable tumor (2, 16, 20) but were most effective in allogeneic tumors (2) or after repeated i.p. immunizations with a subsequent i.p. challenge. Under these conditions, Prager *et al.* (20) reported survival of greater than 60 days in C3H mice that were challenged with the 6C3HED ascites lymphosarcoma. Morgan and Eng (16), working with the same tumor, could only achieve 75% 60-day survivals, despite 3 weekly i.p. injections of chemically-treated cells. Csaba (5) carried out experiments with glutaraldehyde-treated tumor cells in outbred mice. The fixation of I_b tumor cells with formalin has been partially successful in C58 mice challenged with viable I_b leukemia cells (12). More recently, Kudo *et al.* (11) have demonstrated the stabilization of antigen on tumor cells by formaldehyde, but no immunoprophylactic experiments have yet been reported.

MATERIALS AND METHODS

Animals. All experiments were performed with 6- to 10-week-old female BALB/c mice obtained from the breeding unit at the Clinical Research Centre, Harrow, Middlesex, England, or a commercial supplier (Oxford Laboratory Animal Centre, Blackham, Bicester, England).

Tumors. Meth A originated in BALB/c mice (17). This tumor has been maintained by serial passage in mice for a number of years, but it has more recently been stored in 10% dimethyl sulfoxide in medium under liquid nitrogen. Under

[1] This work was supported by a grant from the Medical Research Council, London, England.

[2] Junior Research Fellow, Medical Research Council.

[3] Present address: Department of Immunology and Medicine, Wayne State University School of Medicine, 540 E. Canfield Avenue, Detroit, Mich. 48201.

[4] The abbreviations used are: Meth A, methylcholanthrene-induced sarcoma; SSI, spontaneous adenoacanthoma in BALB/c mice; PBS, phosphate-buffered saline, pH 7.4. This is prepared by adding 6.8 g NaCl, 1.48 g Na_2HPO_4, and 0.43 g KH_2PO_4 to 1 liter double-distilled water. The PBS was autoclaved and the pH was assessed before use.

Received February 18, 1975; accepted June 11, 1975.

these conditions 10^3 tumor cells will grow *in vivo*.

The 2nd tumor (SSI) utilized in these studies arose spontaneously in our colony of BALB/c mice. After removal, the tumor mass was cut into 1-mm sections and was treated with 0.25% trypsin (Difco Laboratories, Detroit, Mich.) in Roswell Park Memorial Institute Medium 1640 at 37° for 1 to 1.5 hr (13). The resulting cells were injected i.p. into syngeneic mice, and the resultant ascitic tumor was reinjected into another group of syngeneic animals. The tumor cells recovered from this 2nd passage were then stored under liquid nitrogen at 2×10^7 cells/ml in 10% dimethyl sulfoxide in Roswell Park Memorial Institute Medium 1640 with 10% fetal calf serum. These 2nd passage cells were passaged into mice 10 days before being used for the challenge of immunized and control mice. These 3rd-passage cells were washed twice before injection. Under these conditions 10^4 cells will grow i.p. and 10^6 cells will grow s.c. Histological examination revealed this tumor to be a mammary adenoacanthoma, a tumor unlikely to contain mammary tumor virus antigens (9).

Glutaraldehyde Treatment. Tumor cells were washed 3 times with PBS pH 7.4, and made up to 10^8/ml in PBS. Glutaraldehyde (25% electron microscopy grade; Taab Laboratories, Reading, England) was diluted to 0.2% in PBS and mixed with an equal volume of the tumor cell suspension. The cells were kept in suspension on a rotator for 10 to 15 min at 22° and then washed 3 times with PBS. The cells were then passed through a muslin pad to remove gross clumps and were resuspended at the required concentration for injection. If these clumps are not removed there is a danger that cells within them will have escaped glutaraldehyde fixation and will remain viable.

Using a modification of the assay for glutaraldehyde described by Frigerio and Shaw (7), it was found that approximately 30 to 50% of the glutaraldehyde was taken up by the cells.

In some experiments the glutaraldehyde-treated cells were further reacted with diketene, 10^{-2} M in borate-0.9% NaCl solution, pH 9.0, or sodium periodate, 10^{-3} M in PBS. In each case the conditions were the same as during the glutaraldehyde treatment.

Glutaraldehyde-treated cells were stored at 4° in PBS containing 0.01% Merthiolate.The stored cells were washed twice in fresh PBS before injection.

Table 1

Effect of a single i.p. immunization on host survival

Groups of 5 mice were given a single i.p. dose of 5×10^7 glutaraldehyde-treated SSI tumor cells and were challenged i.p. 2 weeks later with varied doses of viable SSI tumor cells.

Challenge SSI		No. of survivors			
		Day 20	Day 30	Day 60	Day 120
10^5	Control	0	0	0	0
	Experimental	5	5	5	5
3.2×10^5	Control	0	0	0	0
	Experimental	2	2	2	2
10^6	Control	0	0	0	0
	Experimental	0	0	0	0

Experimental Design. Age- and sex-matched BALB/c mice were given a single injection (unless otherwise specified) of glutaraldehyde-treated tumor cells either i.p. or s.c. Fourteen days later, 5 immunized and 5 control animals were challenged with viable cells (i.p. or s.c.) and were examined every 2 or 3 days for signs of tumor. The cages of the animals were coded so that the examiner could not distinguish between control and experimental groups.

RESULTS

The Protective Effect of Glutaraldehyde-treated Tumor Cells on Subsequent Challenge with Live Tumor

Glutaraldehyde-treated tumor cells protect normal mice against a challenge with viable Meth A or SSI tumor cells. Table 1 shows a representative experiment. Mice immunized with 5×10^7 glutaraldehyde-treated tumor cells i.p. resist a subsequent challenge with 10^5 live cells 2 weeks later. This resistance is dose dependent in that challenge with 3.2×10^5 SSI cells results in only partial protection. Challenge doses of 10^6 cells disclose no protection at all. Although control animals survive as long as 10 to 13 days (depending upon the challenge dose), all show clear evidence of tumor growth (ascites) by Day 7.

Effect of the Dose of Glutaraldehyde-treated Cells

Immunization doses of between 5×10^6 and 10^7 glutaraldehyde-treated cells per animal were only partially protective against a 10^5 challenge. Only doses of 3.2×10^7 or greater gave full protection (Table 2). Similar results were obtained with both fresh and stored glutaraldehyde-treated cells.

Effect of Route of Immunization

i.p. Immunization-s.c. Challenge. With both tumors a single i.p. immunization protected mice against subsequent s.c. challenge (Table 3). A different s.c. challenge dose (10^6)

Table 2

Effect of immunizing dose of glutaraldehyde-treated cells

Groups of 5 mice were immunized i.p. with varying doses of glutaraldehyde-treated tumor cells (SSI) and were challenged 2 weeks later with 10^5 viable tumor cells i.p.

Immunizing dose	Survivors			
	Day 20	Day 30	Day 60	Day 120
Control	0	0	0	0
5×10^4	0	0	0	0
5×10^5	5	0	0	0
5×10^6	3	2	2	2
3.2×10^6	5	1	1	1
10^7	1	1	1	1
3.2×10^7	5	5	5	5
5×10^7	5	5	5	5

Table 3

Effect of i.p. immunization on s.c. challenge

Groups of 5 mice were immunized with 5×10^7 glutaraldehyde-treated tumor cells (i.p.) and were challenged 2 weeks later with viable cells of the same tumor (s.c.).

Challenge	Survivors		
	Day 20	Day 30	Day 60
SSI (10^6)			
Control	2	0	0
Experimental	5	5	5
Meth A (10^5)			
Control	5	0	0
Experimental	5	5	5

Table 4

The effect of s.c. immunization on i.p. challenge

Groups of 5 mice received 1 s.c. injection of 5×10^7 glutaraldehyde-treated tumor cells and were challenged 2 weeks later with 10^5 viable cells of the same tumor given i.p.

Challenge	Survivors		
	Day 20	Day 30	Day 60
SSI			
10^5 Experimental	0	0	0
Control	0	0	0
10^4 Experimental	5	5	5
Control	4	0	0
Meth A			
10^5 Experimental	5	0	0
Control	5	0	0
10^4 Experimental	5	3	3
Control	5	0	0

was used with SSI because lower doses have variable growth in controls.

s.c. Immunization-i.p. Challenge (Table 4). The s.c. immunization route is not as efficient as the i.p. route. The 5×10^7 glutaraldehyde-treated Meth A cells given s.c. protect only partially against a 10^4 challenge. With SSI there is no protection after 10^5 challenge with full protection after 10^4 challenge.

Multiple Immunizations

As many as 3 biweekly immunizations do not alter the dose response against Meth A challenge while only partially increasing protection against SSI (Table 5). Subsequent experiments indicate that even mice that had already rejected a viable challenge cannot reject a challenge dose of 10^6 cells 1 month after the 1st challenge. This implies that while the immune response may be at its maximum, it is inadequate on challenge with high numbers of tumor cells.

Storage

Glutaraldehyde-treated Meth A and SSI tumor cells retain their protective potential even after prolonged storage (Table 6). No difference in dose response of either the immunizing or challenge has been noted.

Effect of Varied Chemical Treatment of Tumor Cells and Their Immunizing Potential

Because glutaraldehyde alone was effective in producing protection against challenge, attempts were made to extend this potential by additional chemical modification. In a long series of experiments performed with the same protocol as that outlined above, it was found that the additional treatment of glutaraldehyde-treated tumor cells with diketene or periodate, or both, did not increase the protection against challenge with viable cells.

Specificity

Experiments have been carried out to test the immunological specificity of the protection afforded by glutaraldehyde-treated cells. (The experiments listed in Table 7 represent 10 experiments of this kind; all of which gave identical results.) Immunization i.p. with 5×10^7 glutaraldehyde-treated syngeneic lymphocytes i.p. gave no protection against an i.p. challenge with 10^5 SSI or Meth A. However, if the number of lymphocytes was increased to 2×10^8 (which has a packed volume similar to that of 5×10^7 tumor cells), protection was observed (Table 7) when both the immunizing and challenge dose were given i.p. Experiments in which

Table 5

Effect of multiple immunizations

SSI[a] i.p. challenge		Survivors		
		Day 20	Day 30	Day 60
10^5	Control	0	0	0
	Experimental	5	5	5
3.2×10^5	Control	0	0	0
	Experimental	5	3	3
10^6	Control	0	0	0
	Experimental	2	1	1

[a] Groups of mice were given 3 i.p. immunizations at 2-week intervals followed 2 weeks later by a challenge with viable cells of the same tumor.

Table 6

Effect of storage on the immunizing potential of glutaraldehyde-treated SSI tumor cells

Groups of 5 mice were immunized i.p. with fresh and stored glutaraldehyde-treated cells and were challenged with 10^5 tumor cells i.p. 2 weeks later.

Duration of storage	Survivors		
	Day 20	Day 30	Day 60
Fresh (same day)	5	5	5
1 wk	5	5	5
11 wk	5	5	5
5 mo.	5	5	4
Control (no immunization)	0	0	0

Table 7

Cross-reacting effect of gluteraldehyde-treated tumor cells

Groups of 5 mice were given injections i.p. or s.c. with 0.2 ml packed volume of glutaraldehyde-treated syngeneic spleen cells or glutaraldehyde-treated SSI or Meth A tumor cells and were challenged 2 weeks later with 10^5 SSI or Meth A tumor cells given i.p.

Immunization	Challenge (i.p.)	Survivors Day 20	Day 30	Day 60
5×10^7 GA[a]-Meth A (i.p.)	10^5 Meth A	5	5	5
5×10^7 GA-Meth A (i.p.)	10^5 SSI	5	5	3, 4[b]
5×10^7 GA-SSI (i.p.)	10^5 Meth A	5	5	4
5×10^7 GA-SSI (i.p.)	10^5 SSI	5	5	5
0.2 ml GA-spleen cells (i.p.)	10^5 SSI	5	4	3, 4[b]
0.2 ml GA-spleen cells (s.c.)	10^5 SSI	0	0	0
Control	10^5 SSI	0	0	0
Control	10^5 Meth A	0	0	0

[a] GA, glutaraldehyde treated.

[b] There were 3 and 4 survivors, respectively, in 2 separate experiments.

the 2 tumors were cross-tested also demonstrated this nonspecificity. However, when immunization and challenge were given by different routes, cross-protection was not observed.

DISCUSSION

The aim of this work was to test the ability of glutaraldehyde to fix tumor antigen while at the same time rendering the cells nonviable. Glutaraldehyde reacts with protein-reactive side-chain groups as an α,β-unsaturated aldehyde and not, as might be expected, as an aldehyde to form the Schiff base (22). The reaction is thus rapid and irreversible under neutral aqueous conditions, and results in intra- and intermolecular cross-linking. Glutaraldehyde has been shown to preserve antigenicity under a variety of conditions. For example, it has been used for preparing immunoadsorbents or soluble proteins (3) and lymphocytes (1). Glutaraldehyde-fixed monolayers have been used as specific adsorbents for cytotoxic T-cells and their precursors (8).

The potential of this approach was also suggested by the observation that the chemical modification of protein by diketene (18) and of erythrocytes by glutaraldehyde (6, 19) reduced the antibody response while maintaining and perhaps enhancing the cellular response. Because of the generally accepted view that cell-mediated reactions play a more important role in tumor rejection than humoral responses, it seemed likely that chemical modification of tumor antigens might be beneficial. The relative role of humoral and cellular immunity in tumor rejection is controversial and in any case we have no evidence to suggest that a relative enhancement of cellular immunity is occurring in our system; nor is there any reason to believe that this is important in the protection we observe in immunized mice.

Our results show that protection against subsequent challenge can be achieved with 2 different BALB/c tumors. One of these (Meth A) has been maintained in serial passage for more than a decade, while the other (SSI) was tested at the 3rd passage from its isolation in our own colony.

Resistance to live tumor challenge is related to the immunizing and challenge dose. Immunization with less than 3.2×10^7 glutaraldehyde-treated tumor cells affords only partial protection, while a challenge with 10^6 viable cells overcomes the response elicited by multiple immunizations. Storage of glutaraldehyde-treated cells for as long as 5 months does not significantly alter their protective potential. Our attempts to further enhance protection by additional modification of protein determinants by diketene or carbohydrate determinants with periodate (19) were not successful.

The prophylaxis observed appears to have a specific immunological as well as a nonspecific component. The nonspecific protection occurs only after i.p. immunization and challenge, in a manner similar to that seen with *Corynebacterium parvum* (4).

Although a nonspecific or adjuvant effect may be an advantage in immunotherapy, the fact that a nonspecific component is present complicates the study of tumor antigenicity. It is possible that much of the nonspecificity derives from the inflammation aroused by residual reactive aldehydes on the treated cells. Further experiments are necessary to see whether this effect can be minimized by blocking these groups. Indeed, it may be possible to utilize them for attaching other strongly immunogenic groups to the cells to enhance the immunogenicity of the tumor antigens (15).

ACKNOWLEDGMENTS

We wish to thank Avril Munro and Geoffrey Taylor, for their technical assistance, and Sir Peter Medawar, for advice and criticism.

REFERENCES

1. Anderson, H. R., and Dresser, D. W. The Preparation and Efficacy of Purified Antilymphocyte Antibody and the Effect of Labelling This Material with ^{125}I. European J. Immunol., *1:* 31–35, 1971.
2. Apffel, C. A., Arnason, B. G., and Peters, J. H. Induction of Tumor Immunity with Tumor Cells Treated with Iodoacetate. Nature, *209:* 694–696, 1966.
3. Avrameas, S., and Ternynck, T. The Cross Linking of Proteins with Glutaraldehyde, and Its Use for the Preparation of Immunoadsorbents. Immunochemistry, *6:* 53–66, 1969.
4. Castro, J. E. Antitumor Effects of *Corynebacterium parvum* in Mice. European J. Cancer, *10:* 121–127, 1974.
5. Csaba, G. Attempts to Induce Antitumor Immunity with Living Attenuated Cells. Neoplasma, *14:* 167–175, 1967.
6. Dennert, G., and Tucker, D. F. Selective Priming of T Cells by Chemically Altered Cell Antigens. J. Exptl. Med., *136:* 656–661, 1972.
7. Frigerio, N. A., and Shaw, M. J. A Simple Method for the Determination of Glutaraldehyde. J. Histochem. Cytochem., *17:* 176–181, 1969.
8. Goldstein, P., Svedmyr, E. A. J., and Blomgren, H. Specific Adsorption of Cytotoxic Thymus Processed Lymphocytes (T cells) on Glutaraldehyde Fixed Fibroblast Monolayers. European J. Immunol., *2:* 380–383, 1972.
9. Hilgers, J. H. M., Theuns, G. J., and Van Nie, R. Mammary Tumor Virus Antigens in Normal and Mammary Tumor-bearing Mice. Intern. J. Cancer, *13:* 568–576, 1973.

10. Klein, G., Sjögren, H. O., Klein, E., and Hellström, K. E. Demonstration of Resistance against Methylcholanthrene-induced Sarcomas in the Primary Autochthonous Host. Cancer Res., *20:* 1561–1572, 1960.
11. Kudo, T., Aoki, T., and Morrison, J. L. Stabilization of Antigens on Surfaces of Malignant Cells by Formalin Treatment. J. Natl. Cancer Inst., *52:* 1553–1557, 1974.
12. Lin, S. L. L., Huber, N., and Murphy, W. H. Immunization of C58 Mice to Line I_b Leukemia. Cancer Res., *29:* 2157–2162, 1969.
13. Madden, R. F., and Burke, D. Production of Viable Single Cell Suspensions from Solid Tumors. J. Natl. Cancer Inst., *27:* 841–861, 1961.
14. Martin, W. J., Wunderlich, J. R., Fletcher, F., and Inman, J. K. Enhanced Immunogenicity of Chemically Coated Syngeneic Tumor Cells. Proc. Natl. Acad. Sci. U. S., *68:* 469–472, 1971.
15. Mitchison, N. A. Immunologic Approach to Cancer. Transplant. Proc., *2:* 92–103, 1970.
16. Morgan, J. F., and Eng, C. P. The Induction of Immunoprotection by Mouse Ascites Tumor Cells Attenuated in Tissue Culture. European J. Cancer, *8:* 293–298, 1972.
17. Old, L. J., Boyse, E. A., Clarke, D. A., and Carswell, E. A. Antigenic Properties of Chemically Induced Tumors. Ann. N. Y. Acad. Sci., *101:* 80–106, 1962.
18. Parish, C. R. Immune Response to Chemically Modified Flagellin. J. Exptl. Med., *134:* 21–47, 1971.
19. Parish, C. R. Preferential Induction of Cell Mediated Immunity by Chemically Modified Sheep Erythrocytes. European J. Immunol., *2:* 143–151, 1972.
20. Prager, M. D., Der, I., Swann, A., and Cotropia, J. Immunization with Chemically Modified Lymphoma Cells. Cancer Res., *31:* 1488–1491, 1971.
21. Prehn, R. T., and Main, J. M. Immunity to Methylcholanthrene Induced Sarcomas. J. Natl. Cancer Inst., *18:* 769–778, 1957.
22. Richards, F. M., and Knowles, J. R. Glutaraldehyde as a Protein Cross Linking Reagent. J. Mol. Biol., *37:* 231–233, 1968.
23. Sanderson, C. J., and Frost, P. The Induction of Tumor Immunity in Mice Using Glutaraldehyde Treated Tumor Cells. Nature, *248:* 690–691, 1974.
24. Thomson, D. M. P., Steele, K., and Alexander, P. The Presence of Tumor Specific Membrane Antigen in the Serum of Rats with Chemically Induced Sarcomas. Brit. J. Cancer, *27:* 27–34, 1973.