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Immunization with Chemically Modified Lymphoma Cells[1]

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SUMMARY

Although 6C3HED ascites lymphosarcoma grows lethally in C3H mice at low tumor cell inoculum, potent immune responses to this tumor have been elicited. It was selected as a model for studying the effectiveness of a number of chemical modifications of the tumor in eliciting immune responses of sufficient potency to protect the animal against a challenging lethal dose of the tumor cells. 6C3HED cells were modified by reactions with: (*a*) diazotized *p*-aminobenzoic acid, (*b*) fluorodinitrobenzene, (*c*) iodoacetamide, (*d*) iodoacetate, (*e*) *N*-ethylmaleimide, and (*f*) *p*-hydroxymercuribenzoate. Cells modified with the strong antigenic determinants *a* and *b* failed to protect against 10^6 6C3HED cells; *c*, *d*, and *e* protected fully; *f* gave partial protection. Alterations producing potent immunity involved blocking sulfhydryl groups with reagents not generally considered good haptens. Iodoacetamide-treated EPF-1 lymphoma protected against an early transplant generation of EPF-1. No cytotoxic antibody to 6C3HED or EPF-1 in immune serum was demonstrable, but lymphoid cells from an immune C3H mouse protected a susceptible animal, indicating cell-mediated immunity.

INTRODUCTION

The concept of stimulating a patient's immune response to his autochthonous tumor with a preparation from that tumor is not a new one. The central problem would seem to entail alteration of the tumor cells so as to minimize or destroy their ability to grow progressively in the host while at the same time preserving antigenicity which is recognizable by the host and to which he can respond. Many early studies involving this approach to cancer therapy were reviewed by Southam (16). The partial success in achieving immunity through use of cells modified by homogenization, freezing and thawing, and heat killing was summarized by McKhann (12). Irradiated cells, incapable of division, have led to immunity in some systems (7, 15), but demonstration (11) that a number of weak histocompatibility antigens are destroyed by X-rays cautions against indiscriminate use of this technique. According to reports by Czajkowski *et al.* (4, 5), significant immune responses to tumor cells modified by attachment of foreign immunoglobulin were obtained; however, Cunningham *et al.* (3) reported disappointing results following use of this approach to enhance immune responses in patients with cancer. McEnany *et al.* (10) encountered inconsistent results when using protein-conjugated tumor cells for immunization against the syngeneic tumor graft. Recently, Martin *et al.* (9) reported enhanced immunogenicity of EL-4 mouse leukemia cells in an *in vitro* system following alteration by either concanavalin A or dinitrophenylaminocaproate. The report of Apffel *et al.* (1) that iodoacetate-treated tumor cells were capable of immunizing a susceptible host against the unaltered tumor has given impetus to our own investigation. Wang and Halliday (17) applied this technique with only partial success in that treated mice demonstrated prolonged survival after challenge but eventually succumbed to growth of the tumor. Jasmin *et al.* (6) also reported a degree of success in protecting mice from Rauscher leukemia cells by prior inoculation with iodoacetamide-treated cells. Knock *et al.* (8) alluded to establishing immunity with cells pretreated with oxophenarsine and 2-(N^4-acetylsulfanilamido)ethyl iodoacetate.

This study was undertaken to determine the efficacy of tumor cells, used as a vaccine following a variety of chemical modifications, in producing immunity to the tumor. Mouse lymphoma 6C3HED was chosen as a model because, although it is lethal in low doses (10^3 cells or less), it is possible to effect immunization (14). Modifications were selected to introduce different groups at various sites in the cell membrane, and a modification producing effective vaccination has been applied to EPF-1 lymphoma, a tumor of recent origin in an early transplant generation. The nature of the immune response was characterized by examination of serum for humoral antibodies and lymphoid cells for ability to protect against unaltered tumor. A preliminary report of part of these findings has been given (13).

MATERIALS AND METHODS

Mice and Tumors. C3H/HeJ and AKR/J mice from Jackson Memorial Laboratory were used in these experiments. Animals were bred in this laboratory by littermate matings. Tumors, maintained by serial transplantation in C3H mice, the strain of origin, include 6C3HED and EPF-1 ascites lymphomas; the EPF-1 lymphoma arose spontaneously during the summer of 1969 and was used in an early transplant generation.

Azobenzoate-modified Cells. For coupling of *p*-aminobenzoate to tumor cells, 6C3HED ascites cells were washed twice with 0.9% NaCl solution and then were suspended in saline buffered with 0.11 M phosphate, pH 7.4, at a concentration of 1.5×10^8 cells/ml. Diazonium salt was prepared by dissolving 0.52 g of *p*-aminobenzoic acid in 10 ml of 1 N HCl; 2.0 ml of this solution were added to 0.53 ml of 10% $NaNO_2$. Reaction was in an ice-salt bath. After 30 min,

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0.1 g of urea was added, and the mixture was then diluted to 3 ml. One ml of the mixture and 1 ml of phosphate-buffered saline were added to 2 ml of cell suspension (0.3 ml packed cell volume). Universal buffer (pH 9) was added as needed to maintain pH 8 to 9. The mixture was kept in the ice-salt bath 1 hr and then refrigerated overnight. Cells were washed 4 times in pH 9 universal buffer and resuspended in 2.7 ml of 0.9% NaCl solution. Because cell residues had become gelatinous, they were homogenized by repeatedly drawing them into a Pasteur pipet.

Cells Modified with FDNB.[2] FDNB, 150 mg in 7.5 ml of acetone, was diluted with 50 ml of 0.05 M Tris-chloride buffer, pH 8.7. Washed tumor cells, packed by centrifugation, were suspended in 10 volumes of 0.9% NaCl solution. Cells were incubated for 30 min at 25° with either 3, 10, 30, or 300 μ-moles of FDNB per ml of packed cells. Cells were washed 2 times with the Tris-buffered 0.9% NaCl solution and were then resuspended (with homogenization, if required) in about 3 volumes of 0.9% NaCl solution. Aliquots were removed for determination of hematocrit and DNP content. The latter was accomplished by dissolution in Hyamine hydroxide and measurement of absorbance at 360 nm. ϵ-DNP-lysine (Sigma Chemical Company, St. Louis, Mo.) dissolved in Hyamine hydroxide served as a standard.

Cells Modified with Sulfhydryl Reagents. Washed tumor cells, packed by centrifugation, were suspended in 10 volumes of 0.11 M phosphate-buffered saline, pH 7.4, containing either iodoacetamide, iodacetate, or *N*-ethylmaleimide at a concentration of 1 to 4 mM. *p*-Hydroxymercuribenzoate (1 mM) was used in 0.005 M phosphate-buffered saline at pH 8.5. The mixture was incubated for 90 min at 37°, and cells were then washed 2 to 3 times with phosphate-buffered saline. If trypan blue staining showed live cells, incubation was continued for up to 4 hr. They were resuspended in 0.9% NaCl solution or Earle's solution for injection.

Vaccination Procedure. Altered cells were resuspended in 0.9% NaCl solution or Earle's solution so that 0.3 ml of suspension delivered the equivalent of 0.1 ml of packed cells (about 10^8 cells). To achieve immunization, weekly injections (usually i.p.) were given for 3 weeks. One week later, mice were challenged with 10^6 unaltered, viable tumor cells of the same type used for immunization and were observed for tumor growth or rejection.

Characterization of the Immune Response to Iodoacetate-modified 6C3HED. C3H mice vaccinated with iodoacetate-modified 6C3HED and surviving challenge with 10^6 6C3HED cells for 7 weeks were rechallenged with about 3 X 10^7 tumor cells. Twelve days later, a suspension of a known number of lymphoid cells prepared from the spleen and lymph nodes of these immunized mice was injected i.p. into nonimmune C3H mice. After 48 hr they were challenged with 5 X 10^5 unaltered, viable cells as determined by exclusion of trypan blue and then observed for tumor growth. The cytotoxic test for humoral antibody performed was essentially the procedure of Boyse *et al.* (2) as used in this laboratory (14).

[2] The abbreviations used are: FDNB, fluorodinitrobenzene; DNP, dinitrophenyl.

RESULTS

Vaccination with Modified 6C3HED. In most experiments, mice were vaccinated 3 times at weekly intervals, and then after 1 additional week were challenged with 10^6 viable tumor cells, a dose which is nearly uniformly lethal. Cells killed by freezing and thawing, heat, or lyophilization were ineffective in conferring protection (Table 1); however, only a single treatment with cells altered by the latter 2 methods was given prior to challenge. In another experiment, 2 injections of lyophilized cells in complete Freund's adjuvant were given intradermally but without benefit. Mice receiving freeze-thaw preparations showed signs of toxicity as indicated by ruffled fur and concave sides. Introduction of azobenzoate groups into the cells was of no value in immunization against unaltered 6C3HED. More extensive studies of cells modified by DNP groups were performed (Table 2). The DNP content varied from 1.7 to 51 μmoles/ml of packed cells (*i.e.*, 1.0 to 30.0 X 10^9 DNP residues per cell, assuming 10^9 cells/ml), but none of these preparations were protective; however, alteration of 6C3HED with the sulfhydryl blocking agents iodoacetamide, iodoacetate, or *N*-ethylmaleimide led to protection (Table 3). Following immunization with cells modified by these 3 reagents and then challenge with the lethal dose of viable tumor, mice remained free of tumor during the period when recurrence might be expected and in several cases during up to 10 months of observation. Because these reagents are at most weak haptens, cells were modified with *p*-hydroxymercuribenzoate, thereby introducing at –SH groups a significant haptenic grouping. An intermediate result was obtained in that experimental animals had prolonged survival but succumbed to tumor. One died of toxicity prior to challenge, while the mean survival of the remaining 5 was 27.4 days. To learn something of the specificity of the vaccination, we gave 3 injections of iodoacetamide-modified C3H mouse spleen cells to C3H mice prior to challenge with 6C3HED. Mean survival was 14.7 days for the control group and 17.2 days for the treated group, a difference without statistical significance ($p = 0.3$).

Characterization of the Immune Response of C3H Mice to Iodoacetate-modified 6C3HED. Cytotoxic tests were performed to determine whether humoral antibody was involved in the immunity to 6C3HED in C3H mice that resisted 2 challenges of the tumor following vaccination with iodoacetate-modified cells. Although tests were repeated at various times following induction of immunity, no cytotoxic antibody was demonstrable. However, immunity could be

Table 1
Vaccination of C3H mice with altered 6C3HED cells

Treatment	No. of mice	Mean survival (days)
None	15	15
Heat, 56°[a]	10	15
Freeze-thaw	8	9
Lyophilization[a]	8	13
Diazotized *p*-aminobenzoic acid	6	13

[a] Single immunizing dose; 3 immunizing doses for others.

Table 2
Vaccination of C3H mice with DNP-6C3HED

Reaction mixture (μmoles FDNB/ml cells)	Product (μmoles DNP/ml cells)	No. of mice	Survival (days)
3	1.7	5	13.0 ± 0.45[a]
10	5.1	5	12.8 ± 0.37
30	11.5	5	12.8 ± 0.37
300	51.0	3	15.7 ± 1.76
No treatment		5	14.5 ± 0.40

[a] Mean ± S.E.

Table 3
Vaccination of C3H mice with 6C3HED modified by –SH reagents

Reagent	No. of mice	Survival (days)
None	38	15.4 ± 0.38[a]
Iodoacetamide	6	>60
	10	>90
Iodoacetate	6	>60
N-Ethylmaleimide	6	>90
p-Hydroxymercuribenzoate	6	27.4[b] ± 6.86[a]

[a] Mean ± S.E.
[b] For 5 mice; 6th died prior to challenge with 6C3HED.

passed with lymphoid cells from spleen and lymph nodes of immune animals. Experimental animals receiving a suspension of either 5 X 10^7 or 5 X 10^6 immune lymphoid cells were protected when challenged 48 hr later with 5 X 10^5 viable 6C3HED cells.

Modification of EPF-1 and Characterization of the Immune Response. The EPF-1 lymphoma has many features in common with 6C3HED, and the immune response of C3H mice to this tumor under a number of conditions has been found to be similar (M. D. Prager, I. Derr, and D. W. Woodridge, manuscript in preparation). Homogenized, but otherwise untreated EPF-1 cells were toxic to C3H mice who died 10 days after an i.p. injection of the homogenate. Three injections of iodoacetamide-modified EPF-1 cells successfully immunized all C3H mice against EPF-1 (10^6 cells) (Table 4); a single injection of iodoacetamide-modified EPF-1 (10^8 cells) protected only one-half of the animals. The mean survival time of the 6 mice that died with tumor was 25.7 days compared to 14.3 days for the control group. Mice surviving after either 1 or 3 vaccinations resisted further challenge with 3 X 10^7 EPF-1 cells, but no cytotoxic antibody to the tumor could be demonstrated in their serum.

Response of AKR Mice to Iodoacetamide-modified 6C3HED. AKR and C3H mice are identical at the *H-2* histocompatibility locus (*H-2*^{k}), but they differ at minor histocompatibility loci. It therefore seemed of interest to compare the responses of the 2 mouse strains. In other experiments (M. D. Prager, I. Derr, and D. W. Woodridge, manuscript in preparation), it was shown that AKR mice immunized to 6C3HED develop a circulating, cytotoxic antibody. Iodoacetamide-modified 6C3HED cells were given at weekly intervals; 5 days after the 2nd vaccination, no cytotoxic antibody was detectable, but 5 days after the 3rd injection antibody was demonstrable, and mice were then resistant to challenge with an otherwise lethal dose of 30 X 10^6 6C3HED cells.

DISCUSSION

Results with the 2 lymphomas, 6C3HED and EPF-1, clearly indicate that it is possible to achieve potent immunity to these tumors in the mouse strain in which they originated (*i.e.*, C3H) by use of chemically modified 6C3HED and EPF-1 cells, respectively. Cells killed by heat, freezing and thawing, or lyophilization failed to immunize. Cells modified with either azobenzoate or DNP groups were also ineffective immunogens for the native tumor cells. Azobenzoate attaches primarily at tyrosine residues in the cell membrane and DNP at amino groups. It is probably significant that both of these alterations introduce strongly haptenic groups, and antigenic competition may be involved in their lack of effectiveness; *i.e.*, if the specificity of the immune response is directed primarily against the hapten, no response effective against unmodified tumor cells may occur. Variation of the degree of substitution with DNP groups from 51 to 1.7 μmoles/ml of packed 6C3HED cells failed to produce an agent useful for immunization. Reagents effective in immunizing C3H mice to these tumors were iodoacetamide, iodoacetate, and *N*-ethylmaleimide. These reagents react with sulfhydryl groups, and they are not good haptens. To test whether both features are important, cells were altered with *p*-hydroxymercuribenzoate, a reagent which reacts with sulfhydryl groups but which introduces essentially the same haptenic group introduced by diazotized *p*-aminobenzoic acid. Mice not succumbing to the toxicity of this treatment demonstrated prolonged survival but eventually died with proliferating tumor. Since these mice survived longer than those receiving azobenzoate-modified cells, sulfhydryl substitution appears beneficial. However, since these mice did less well than those receiving cells modified by the other –SH reagents, it also appears important to avoid introducing groups that serve as strong antigenic determinants and thereby provide antigenic competition.

Immunity to 6C3HED following vaccination with iodoacetate-modified cells is cell mediated. Evidence is supplied by the protection afforded nonimmune C3H mice challenged with a lethal dose of 5 X 10^5 6C3HED tumor cells following prior injection of 10 to 100 times that number of lymphoid cells from an immune C3H mouse. No cytotoxic antibody was demonstrable in C3H mouse serum to either

Table 4
Protection of C3H mice from EPF-1 lymphoma by iodoacetamide-modified EPF-1

No. of vaccinations	No. of mice	Survivors (%)	Survival of dead mice (days)
0	8	0	14.3 ± 0.25[a]
1	12	50	25.7 ± 3.70
3	10	100	

[a] Mean ± S.E.

6C3HED or EPF-1. The 3-dose immunization schedule appears to be a reasonable one because a single injection of modified EPF-1 cells protected only 50% of C3H mice from a challenging dose of the tumor, and cytotoxic AKR mouse antibody to 6C3HED appeared after the 3rd dose of altered cells but was not detected after the 2nd. The latter observation also indicates that the humoral response to the modified cells is effective against unaltered 6C3HED.

REFERENCES

1. Apffel, C. A., Arnason, B. G., and Peters, J. H. Induction of Tumour Immunity with Tumour Cells Treated with Iodoacetate. Nature, *209:* 694–696, 1966.
2. Boyse, E. A., Old, L. J., and Thomas, G. A Report on Some Observations with a Simplified Cytotoxic Test. Transplant. Bull., *29:* 435–438, 1962.
3. Cunningham, T.J., Olson, K.B., Laffin, R., Horton, J., and Sullivan, J. Treatment of Advanced Cancer with Active Immunization. Cancer, *24:* 932–937, 1969.
4. Czajkowski, N.P., Rosenblatt, M., Cushing, F.R., Vazquez, J., and Wolf, P.L. Production of Immunity to Malignant Neoplastic Tissue. Cancer, *19:* 739–749, 1966.
5. Czajkowski, N.P., Rosenblatt, M., Wolf, P.L. and Vazquez, J. A New Method of Active Immunization to Autologous Human Tumour Tissue. Lancet, *2:* 905–909, 1967.
6. Jasmin, C., Piton, C., and Rosenfeld, C. Effets de l'Iodoacetamide sur les Cellules de la Leucemie Virale de Rauscher. Intern. J. Cancer, *3:* 254–259, 1968.
7. 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.
8. Knock, F.E., Galt, R.M., and Oester, Y.T. Protein-Sulfhydryl Groups in Cellular Control Mechanisms and Cancer. J. Am. Geriat. Soc., *15:* 882–899, 1967.
9. 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.
10. McEnany, M. T., Kelly, M. G., and Fahey, J. L. Effects of Immunization with Tumor Cell-Foreign Protein Conjugates on Growth of Syngeneic Tumor Grafts. Proc. Am. Assoc. Cancer Res., *9:* 46, 1968.
11. McKhann, C.F. The Effect of X-ray on the Antigenicity of Donor Cells in Transplantation Immunity. J. Immunol., *92:* 811–815, 1964.
12. McKhann, C.F. Methods of Detecting Cancer Antigens and Antitumor Antibody. Federation Proc., *24:* 1033–1036, 1965.
13. Prager, M.D., Derr, I., Swann, A., and Cotropia, J. Immunization with Chemically Modified Cancer Cells. Proc. Am. Assoc. Cancer Res., *12:*2, 1971.
14. Prager, M.D., Roberts, J., and Bachynsky, N. Immunity to the 6C3HED Ascites Tumor following Treatment of Tumor-bearing Mice with *Escherichia coli* L-Asparaginase. J. Immunol., *98:* 1045–1052, 1967.
15. Revesz, L. Detection of Antigenic Differences in Isologous Host-Tumor Systems by Pretreatment with Heavily Irradiated Tumor Cells. Cancer Res., *20:* 443–451, 1960.
16. Southam, C.M. Applications of Immunology to Clinical Cancer. Past Attempts and Future Possibilities. Cancer Res., *21:* 1302–1316, 1961.
17. Wang, M., and Halliday, W.J. Immune Responses of Mice to Iodoacetate-treated Ehrlich Ascites Tumour Cells. Brit. J. Cancer, *21:* 346–352, 1967.