PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 145, 636–640 (1974)

# Failure of Shier's Chemical Vaccine to Protect BALB/c Mice Against Transplant and Chemically Induced Tumors[1] (37865)

M. M. COOK, A. F. WAGNER, V. M. LARSON, A. A. TYTELL, T. Y. SHEN, AND M. R. HILLEMAN

*Division of Virus and Cell Biology Research, Merck Institute for Therapeutic Research, West Point, Pennsylvania 19486, and Synthetic Chemical Research, Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065*

Most neoplastic cells are more agglutinable by plant lectins such as wheat germ agglutinin than are normal cells. This is believed to be due to an increase in number or accessibility of lectin binding sites or receptors on the cell surface. Burger (1) showed that the receptor is a soluble glycoprotein containing *N*-acetylglucosamine. Shier (2) prepared a chemical vaccine against cancer by condensing di-*N*-acetylchitobiosylamine with sodium poly(L-aspartate). The synthetic receptor was then complexed with methylated bovine serum albumin and incorporated into Freund's complete mineral oil adjuvant. Test vaccine containing di-*N*-acetylchitobiosyl poly(L-asparagine) and control vaccine containing poly(L-aspartate) not linked to the disaccharide were used to immunize BALB/c mice against challenge with XS63·5 or MOPC 70A myeloma of BALB/c mice or with 3-methylcholanthrene. Shier reported that his vaccine retarded tumor development of both kinds and suggested that further modification of the preparation might ultimately lead to the development of a vaccine that would elicit a strong protective cellular immune response with a negligible humoral response.

The sound theoretical basis for Shier's vaccine and the reported success were regarded to be of such importance as to warrant careful repetition employing exactly the same methods as were used by Shier as published (2) and elucidated in personal communications. Such studies were done and no evidence for protective effect was obtained. The results in the experiments are presented here.

*Materials and Methods. Vaccine.* The di-*N*-acetylchitobiosyl poly(L-asparagine) (Shier antigen A) was prepared according to Shier (2) with the exception that sodium azide in dimethylformamide was substituted for silver azide in the step involving conversion of the glycosyl halide intermediate to the corresponding azide (3). Two such preparations were made. Preparation 1 contained 11.1% of disaccharide and preparation 2 14.0% which was in the same range as the preparations made by Shier (2). The methylated bovine serum albumin (MBSA) (Worthington Biochemicals, Co., Freehold, NJ) was complexed with the di-*N*-acetylchitobiosyl poly(L-asparagine) preparations according to Shier (2). Freund's adjuvant consisted of 45 ml Drakeol 6VR mineral oil (Pennsylvania Refining Co., Butler, PA), 5 ml Arlacel A emulsifier (Hilltop Laboratories, Cincinnati, OH), and 2 mg/ml of killed *Mycobacterium tuberculosis* organisms (Difco Laboratories, Detroit, MI). Three volumes of the adjuvant and 1 vol of the di-*N*-acetylchitobiosyl poly(L-asparagine)–MBSA complex were emulsified by reciprocal expelling through two interconnected syringes. All reagents were stored at 4°, and fresh emulsion was prepared just before use in every instance. Control antigen was iden-

[1] This work was supported in part by Contract No. NIH-NCI-E-71-2059 within The Virus Cancer Program of the National Cancer Institute.

TABLE I. Immunization and Challenge Schedules for Testing Shier's di-*N*-acetylchitobiosyl poly(L-asparagine) Vaccine (Antigen A).

| Vaccination | | Test system | | | | | |
|---|---|---|---|---|---|---|---|
| | | XS63·5 Tumor transplant | | | 3-Methylcholanthrene tumor induction | | |
| | | Vaccination | | Challenge | Vaccination | | Challenge |
| Schedule (Shier) | Amount of antigen A per dose ($\mu$g) | Route[a] (groin) | Age of mouse (weeks) | Age of mouse (weeks) | Route (groin) | Age of mouse (weeks) | Age of mouse (weeks) |
| I | 0.25 | sc × 2[b] | 6 | | — | — | — |
| | 0.25 | sc × 2 | 8 | 12 | — | — | — |
| II | 2.5 | ip and sc × 2 | 6 | | — | — | — |
| | 2.5 | sc × 2 | 8 | | — | — | — |
| | 2.5 | sc × 2 | 10 | 12 | — | — | — |
| VI | 0.25 | sc × 2 | 6 | 9 | — | — | — |
| IV | 2.5 | — | — | — | sc × 2 | 6 | 6 |
| | 2.5 | — | — | — | sc × 2 | 9 | — |
| V | 12.5 | — | — | — | ip and sc × 2 | 6 | 6 |
| | 12.5 | — | — | — | sc × 2 | 8 | — |
| | 12.5 | — | — | — | sc × 2 | 10 | — |

[a] sc = subcutaneous; ip = intraperitoneal.

[b] Two doses, each given subcutaneously into the region of each groin.

tical to vaccine antigen except that the poly(L-aspartate)–MBSA (Shier antigen B) was substituted for the di-*N*-acetylchitobiosyl poly(L-asparagine)–MBSA complex.

*Wheat germ agglutinin (WGA) assay.* Wheat germ agglutinin was prepared by the method of Burger and Goldberg (4), and the assay for inhibition of agglutination of sheep erythrocytes was performed as described by Shier (2). Four units of agglutinin were employed in the tests.

*Mice.* Male and female BALB/c mice were obtained from Microbiological Associates, Inc., through the courtesy of the Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, Bethesda, MD, and were used at 6 weeks of age.

*Tumor cell line.* The XS63·5 BALB/c myeloma cell line was obtained from the same source as Shier, Dr. M. Cohn of the Salk Institute, San Diego, CA. The cells were maintained by weekly serial culture employing Dulbecco's modified Eagle's medium supplemented with 10% horse serum and 11.7 $\mu$g/ml of glutamine (2).

*Carcinogen.* 3-Methylcholanthrene (3-MC) was purchased from Mann Research Laboratories, NY, and prepared at a concentration of 0.5 mg/0.1 ml in olive oil.

*Experimental design.* Groups of 24 mice were given test vaccine (antigen A) according to Shier's (2) regimens as shown in Table I. The control animals received antigen B by the same regimen. In the transplant challenge system, groups of 6 vaccinated (antigen A) and 6 control (antigen B) mice were challenged intraperitoneally with $10^5$, $5 \times 10^5$, $10^6$, or $10^7$ XS63·5 tumor cells in 0.2 ml Dulbecco's modified Eagle's medium. The mice were observed daily for appear-

TABLE II. Inhibition of Agglutination of Sheep Erythrocytes by Wheat Germ Agglutinin.

| Compound | Relative inhibitory activity on a weight basis |
|---|---|
| *N*-Acetylglucosamine | 1 |
| Di-*N*-acetylchitobiosylamine | 10 |
| Di-*N*-acetylchitobiosyl poly(L-asparagine) | |
| Preparation 1 | 320 |
| Preparation 2 | 320 |
| Sodium poly(L-aspartate) | 0 |

TABLE III. Evaluation of Shier's di-*N*-acetylchitobiosyl poly(L-asparagine) Vaccine for Protective Efficacy.

| Vaccination | | | Tumor development at 9 weeks[a] — XS63·5 tumor cell challenge | | | | |
|---|---|---|---|---|---|---|---|
| | | | $1 \times 10^5$ cells | $5 \times 10^5$ cells | $1 \times 10^6$ cells | $1 \times 10^7$ cells | |
| Schedule (Shier) | Antigen preparation No. | Material given | No. with tumor/ Total No. | No. with tumor/ Total No. | No. with tumor/ Total No. | No. with tumor/ Total No. | Average day of tumor detection |
| I | 1 | Vaccine | 0/5 | 0/5 | 2/6[b] | 5/5 | 10.6[c] |
| | | Control | 0/6 | 1/6 | 1/6 | 5/6 | 10.4 |
| | 2 | Vaccine | 0/6 | 0/6 | 2/5 | 6/6 | 17.8[c] |
| | | Control | 0/6 | 0/5 | 2/6 | 6/6 | 14.5[c] |
| II | 1 | Vaccine | 1/6 | 1/5 | 2/4[d] | 5/5 | 17.7 |
| | | Control | 1/6 | 1/6 | 2/6 | 4/5 | 20.3 |
| | 2 | Vaccine | 2/6 | 2/6 | 0/6 | 3/5 | 14.0 |
| | | Control | 1/6 | 1/6 | 0/5 | 4/6 | 15.3 |
| VI | 1 | Vaccine | 1/5 | 0/6 | 2/5 | 5/6 | 11.0 |
| | | Control | 0/6 | 0/6 | 2/5 | 5/6 | 10.7 |
| | 2 | Vaccine | 0/6 | 0/6 | 3/6 | 4/5 | 13.6 |
| | | Control | 1/6 | 0/5 | 0/6 | 2/6 | 20.7 |

| | | | Tumor development at 20 weeks — 0.5 mg 3-MC challenge | |
|---|---|---|---|---|
| | | | No. with tumor/ Total No. | Average day of tumor detection |
| IV | 1 | Vaccine | 15/21 | 100.7 |
| | | Control | 21/23 | 97.1 |
| | 2 | Vaccine | 15/24 | 111.7 |
| | | Control | 15/22 | 113.5 |
| V | 1 | Vaccine | 17/24 | 116.1 |
| | | Control | 14/22 | 118.2 |
| | 2 | Vaccine | 18/23 | 108.5 |
| | | Control | 11/23 | 111.2 |

[a] Schedule I, preparation No. 2, 8 weeks.
[b] Two additional tumors appeared at Days 10 and 15 and regressed by Day 63.
[c] Average days to tumor death.
[d] One additional tumor appeared at Day 18 and regressed by Day 34.

ance of tumors and for tumor death for 9 weeks. In the chemical challenge experiments, 0.1 ml (0.5 mg) of 3-MC was inoculated intramuscularly in the region of the right scapula at the time of the first dose of vaccine. The mice were palpated twice weekly for tumors for 14 weeks beginning 6 weeks after challenge.

*Results. Assay for inhibition of wheat germ agglutinin.* The biological activity of the di-*N*-acetylchitobiosylamine-containing preparations was established in tests for their ability to inhibit hemagglutination by WGA as shown in Table II. The values were similar to those reported by Shier (2) for his preparations. Essentially, the tests showed that there was a high affinity of the chitobiose materials for the WGA and that the di-*N*-acetylchitobiosyl poly(L-asparagine) was far more active than the disaccharide alone. The poly(L-aspartate) had no affinity for the WGA.

*Assay for protective efficacy of Shier's vaccine.* Shier's antigen A vaccine was assayed for capacity to immunize against XS63·5 tumor given by transplant or against 3-MC-induced tumors. The regimens were as shown in Table I. Both preparations 1 and 2 of Shier's antigen A, with the appropriate control antigen B, were tested by each of these regimens. The results presented in Table III show no significant protection by the vaccine against XS63·5 transplant tumor at any challenge dose level used. The average time period for tumor occurrence was the same in the vaccine and control animals. The rates for tumor development were not significantly different in any instance. The findings in additional control tests not reported here in which the animals were given diluent in Freund's adjuvant or tumor cells only were essentially the same as for the tests reported in the table.

Table III also shows that there was no significant suppression in 3-MC tumor incidence or delay in time of tumor occurrence in the groups immunized with Shier's antigen A as compared to the controls. Figure 1 presents the findings in greater detail and substantiates the conclusion that the vaccine was without significant effect.

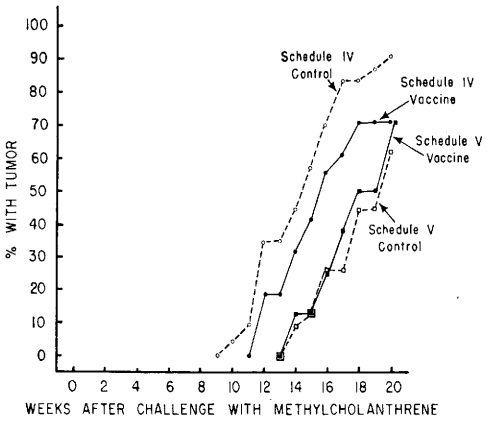


FIG. 1. Antigen preparation 1. Development of tumors induced by 3-methylcholanthrene in mice immunized with Shier's antigen A (di-*N*-acetylchitobiosyl poly(L-asparagine)) and control antigen B (poly(L-aspartate)).

*Discussion.* Shier presented preliminary evidence to indicate that his chemical vaccine retarded tumor progression in transplant and chemically induced cancer. In the transplant challenge system using only a small number of mice (2/group), 2 of 4 sets of data showed that the survival time of mice that had received antigen A was significantly greater ($P < .05$) than in those mice treated with control antigen B. In his tests for prevention of methylcholanthrene-induced tumor, larger numbers of animals were used. In 1 of the 2 experiments, there was a statistically significant delay in time of tumor appearance in animals that received antigen A.

The present experiments were carried out in larger numbers of animals with two different preparations of antigen A and employed the same methods for making vaccine and the same test systems as used by Shier (2). The vaccine appeared clearly without effect either in delaying appearance or in preventing transplant and methylcholanthrene-induced cancer.

*Summary.* Chemical vaccine containing di-*N*-acetylchitobiosyl poly(L-asparagine) prepared as described by Shier was assayed for its ability to prevent XS63·5 myeloma transplant tumors and 3-methylcholanthrene-induced tumors in BALB/c mice. The vaccine was ineffective.

The authors are indebted to W. Clark, T. Napier, W. McGuire, J. Rotz, G. Lampson, W. Fisher, and R. Bugianesi for technical assistance and to A. McLean for statistical analyses.

1. Burger, M. M., Nature **219**, 499 (1968).

2. Shier, W. T., Proc. Nat. Acad. Sci. USA **68**, 2078 (1971).

3. Spinola, M., and Jeanloz, R. W., J. Biol. Chem. **245**, 4158 (1970).

4. Burger, M. M., and Goldberg, A. R., Proc. Nat. Acad. Sci. USA **57**, 359 (1967).

Received Sept. 4, 1973. P.S.E.B.M., 1974, Vol. 145.