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Preparation of a "Chemical Vaccine" Against Tumor Progression

[substituted glucosaminyl poly(L-aspartate)]

W. THOMAS SHIER

The Armand Hammer Center for Cancer Biology, The Salk Institute, Post Office Box 1809, San Diego, California 92112

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ABSTRACT **A synthetic antigen containing the presumed receptor site for wheat-germ agglutinin (a lectin capable of specifically agglutinating tumor cells) elicits an immune response in mice capable of cross-reacting with receptor sites for wheat-germ agglutinin on tumor-cell surfaces. Mice immunized against the antigen in complete Freund's adjuvant are able to reject five times as many transplanted myeloma tumor cells as are rejected by otherwise identically treated control mice. Methylcholanthrene-induced tumors appear later in mice immunized against the antigen than in controls. The protection of mice against tumor transplants can be transferred to unimmunized mice with spleen cells, but not with serum from immunized donors. Immunization of mice against the wheat-germ agglutinin receptor seemed to have no marked deleterious effect on biological processes in normal dividing cells.**

Evidence has been accumulating over a period of many years to indicate that the primary host defense against malignancy is a cell-mediated immune response mounted against tumor-specific surface antigens (1). However, the promise of immunotherapy for the control of cancer has resisted practical exploitation because of two problems. The first problem is immunological enhancement of tumor growth: if a humoral immune response, especially that of IgG, is mounted against the surface antigens, it blocks the action of the cellular immune system, and the rate of growth of the tumor is enhanced (2). The second problem is that the number of distinct tumor-specific surface antigens is very large (3), so that immunization against one tumor type affords little or no protection against all other tumor types (4).

In contrast to this diversity, virtually all tumor cell lines can be agglutinated about ten times as effectively as normal cell lines by a lectin, wheat-germ agglutinin (5, 6) (WGA). As a result of studies on the binding of fluorescein-labeled WGA to fibroblasts, Box *et al.* (7) have interpreted the specific agglutination in terms of WGA receptor sites present on the surface of tumor cells at all times, but present on the surface of normal cells only during cell division.

In this paper I report the application of the so-called "chemical vaccine" approach of immunoparisitology (8) to the cancer problem. As strictly applied to the cancer problem, this approach would involve isolating from the surfaces of tumor cells the antigenic determinants of the tumor-specific surface antigens, determining their structures, and synthesizing them in an immunogenic form. However, the discovery of a WGA receptor site common to virtually all tumor cells suggests a solution to the problem caused by the diversity of the surface antigens. The results presented in this paper, although preliminary, indicate that the WGA receptor sites can be used as tumor antigens.

From agglutination-inhibition studies performed by Burger, one can infer the chemical structure of the receptor site for which WGA is specific (5). The agglutination of tumor cells by WGA was inhibited by *N*-acetylglucosamine (GlcNAc) and about ten times as effectively, on a molar basis, by the disaccharide of GlcNAc, di-*N*-acetylchitobiose (5). However, *N*-acetylchitodextrins (higher homologs of GlcNAc) are not more effective on a molar basis than di-*N*-acetylchitobiose (9), which suggests that WGA is specific only for the disaccharide. Burger (10) has isolated the WGA receptor site from the surface of tumor cells, and shown it to be a soluble glycoprotein containing GlcNAc.

In all soluble glycoproteins that have been studied to date, when GlcNAc is found at the carbohydrate–protein linkage, it is linked β-D-glucosidically to the amide group of an L-asparagine (11). Presumably the same linkage is found in the WGA receptor protein, and hence the receptor site has the partial structure shown in Fig. 1.

I have prepared a synthetic antigen designed to elicit an immune response able to crossreact with the WGA receptor site on tumor cells. Preliminary evidence indicates that progression of tumors of a wide range of antigenic specificity is retarded in mice immunized with this antigen. It is possible that modification of the antigen, adjuvant, and immunization schedule will ultimately permit the development of a vaccine that will elicit a strong protective cellular immune response with a negligible humoral response.

MATERIALS AND METHODS

WGA (Miles) was purified by the method of Burger and Goldberg (5). 3-Methylcholanthrene was obtained from Sigma. Complete Freund's adjuvant was prepared by mixing Markol-52 (100 ml, Humble Oil and Refining Co.), Aquaphor (50 ml, Duke Laboratories), and killed *Mycobacterium butyricum* (400 mg, Difco). Di-*N*-acetylchitobiose was prepared by de-*O*-acetylation of octaacetylchitobiose (see below) in ammonia-saturated absolute methanol. Sheep erythrocytes were obtained from Flow Laboratories.

Agarose diffusion assays were performed in 1% agarose (Calbiochem) on microscope slides. Hemagglutination and hemagglutination inhibition were assayed in a Takatsy microtitrator apparatus (Microtiter, Cooke Engineering Co., Alexandria, Va.), with 25 μl of phosphate-buffered saline per well as diluent for 2-fold dilutions of 25 μl of test sample, and 25 μl of a suspension of 2×10^8 sheep erythrocytes per ml.

Abbreviations: WGA, wheat-germ agglutinin; ECDI, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide hydrochloride; MBSA, methylated bovine-serum albumin.

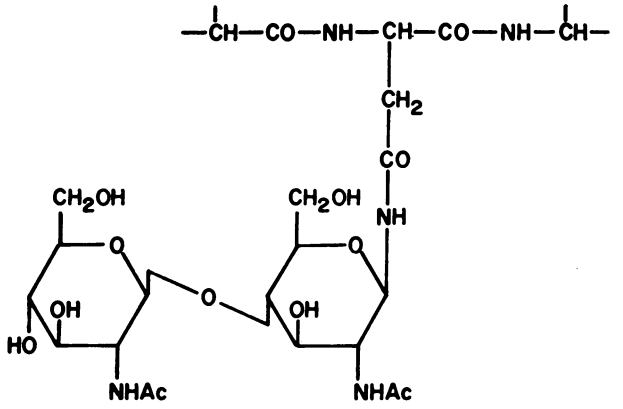

FIG. 1. Proposed partial structure of the WGA receptor site.

The BALB/c myeloma cell lines XS63·5 and MOPC 70A were obtained from Dr. M. Cohn, and cultured in Dulbecco's modification of Eagle's medium supplemented with 10% horse serum (Grand Island Biological Co.) and 11.7 μg/ml of glutamine. BALB/c mice, 4–8 weeks old, were obtained from our own colony, and used in age- and sex-matched groups. Mice were challenged with a single intraperitoneal injection of tumor cells suspended in 0.2 ml of Dulbecco's modified Eagle's medium. Mice showing no evidence of an XS63·5 tumor after 60 days were considered to have caused a complete regression of the challenge tumor; there has been no incidence and death from an ascites tumor observed later than 39 days after challenge.

The following immunization schedules have been used: I, The antigen solution (10 μg/ml) was emulsified in 3 volumes of complete Freund's adjuvant; a subcutaneous injection of 0.1 ml in each groin was repeated at 2 weeks and the mice were challenged at 6 weeks. II, The antigen solution (100 μg/ml) was processed as in I; an intraperitoneal injection of 0.1 ml as well as a subcutaneous injection of 0.1 ml in each groin was followed at 2 and 4 weeks with subcutaneous injections of 0.1 ml in each groin, and the mice were challenged at 6 weeks. III, The same as schedule II, except that no intraperitoneal injection was administered. IV, The same as schedule I, except that antigen solutions at 100 μg/ml were used and the booster injections were given at 3 weeks. V, The same as schedule II, except that antigen solutions at 500 μg/ml were used. VI, The same as schedule I, except that there was no boost at 2 weeks and the mice were challenged at 3 weeks.

Pooled spleen-cell suspensions were prepared immediately after removal of the spleens by teasing the spleens apart in Eagle's medium, and passing the resulting suspension through a sterile 200-mesh stainless steel screen. The number of viable nucleated cells was counted at a 1:100 dilution in 0.05% Trypan blue (Matheson, Coleman and Bell) in 2% acetic acid in saline. The number of direct plaque-forming cells was determined by the method of Jerne and Nordin (12). The number of rosette-forming cells was determined by the method of Cone and Johnson (13) using suspensions of 6×10^6 spleen cells/ml and 48×10^6 sheep erythrocytes per ml.

Preparation of the antigens

Octaacetylchitobiose, mp 308–309°C (dec.) (ref. 14, 308–309°C [dec.]) was prepared by partial acetolysis of crustacean chitin (Calbiochem) by the method of Barber *et al.* (14), and converted to 2-acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-3,6-di-*O*-acetyl-2-deoxy-β-glucopyranosyl azide, mp 187–189°C (dec.) (ref. 15, 198–199°C [dec.]), via the glycosyl bromide by the method of Bolton and Jeanloz (16). A better synthesis of this intermediate has subsequently appeared in the literature (15). The glycosyl azide was de-*O*-acetylated by the method of Michael and Wulff (17), and the crude product was hydrogenated over platinum black (16) to yield 2-acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-β-D-glucopyranosylamine (compound I, mp 190–192°C; R_f in 1-butanol–pyridine–water 6:4:3, 0.1).

The antigen, *A*, was prepared as follows: sodium poly(L-aspartate) [34.5 mg, prepared from poly(L-aspartic acid) (Sigma) by dissolving a sample in 0.1 M sodium carbonate followed by dialysis and lyophilization], compound I (120 mg), ethylenediamine (0.1 mg), and the coupling compound 1-ethyl-3(3-dimethylaminopropyl)-carbodimide hydrochloride (ECDI) (100 mg, Sigma) were dissolved in 1 ml of water and allowed to stand at room temperature for 1 week. The solution was dialyzed against 10% NaCl and then water, and lyophilized to yield 33.5 mg of *A* containing about 13% substitution of available carboxyl groups as determined by amino-acid analysis. The control antigen, *B*, was prepared in an identical manner except that no compound I was added to the reaction mixture. The complexes of *A* and *B* with methylated

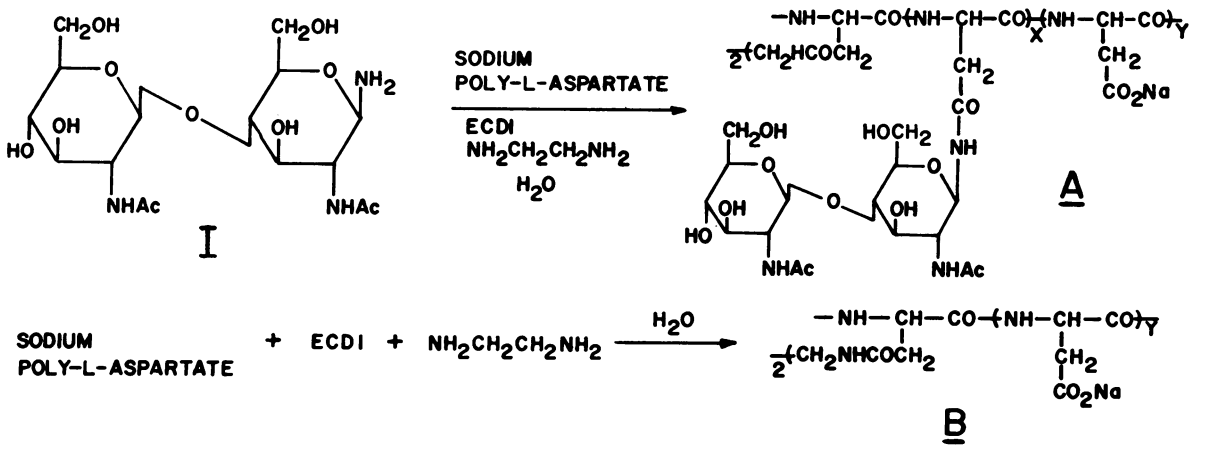

FIG. 2. Synthesis of the antigen, *A*, and the cross-linked polypeptide backbone, *B*.

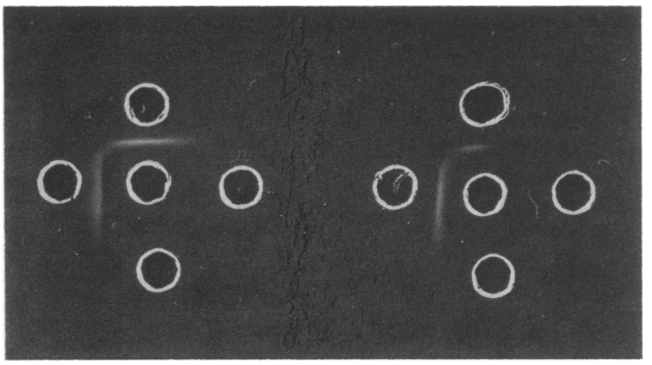

FIG. 3. WGA–*A* precipitation in agarose. In both sets of wells a solution of WGA was placed in the center well, *A* (15 mg/ml) in the left and upper wells, and *B* (15 mg/ml) in the right and lower wells. The plate was allowed to stand overnight, during which time the precipitin lines formed. 1 hr before the photograph was taken, a saturated solution of di-*N*-acetylchitobiose was added to the extreme right well; part of the adjacent precipitate line has dissolved.

bovine serum albumin (MBSA, Sigma) were prepared by dropwise addition of a 1% solution of the latter to 20 volumes of a stirred solution of the antigen in water (0.5 mg/ml). The resulting suspension was sonicated to disperse the precipitate and diluted.

RESULTS

One synthetic antigen that could be expected to elicit an immune response against the partial structure given in Figure 1 is composed of poly(L-aspartic acid) having some of the free carboxyl groups substituted with a sugar moiety. The simplest way to prepare such an antigen (18) is to link the glycosylamine via a peptide linkage to free carboxyls of poly(L-aspartate) (Fig. 2). In practice, up to 13% of available carboxyl groups were substituted with the sugar moiety. Ethylenediamine was added to the reaction mixture as a cross-linking agent intended to increase the molecular weight and therefore the immunogenicity of the antigen, *A*. The cross-linked polypeptide backbone, *B*, was prepared under identical conditions for use as a control antigen.

Two types of experiments indicate that WGA has a specific affinity for *A*. As shown in Fig. 3, WGA, *A*, and di-*N*-acetylchitobiose interact like antibody, antigen, and hapten, respectively, in an agar diffusion assay. This experiment, and hemagglutination inhibition data (Table 1), establish that

TABLE 1. *Inhibition of agglutination of sheep erythrocytes by WGA*

Inhibitor	Relative inhibition*
N-Acetylglucosamine	1
Di-*N*-acetylchitobiose	15
A	240
B	0

* Inhibition of agglutination of sheep erythrocytes by 50 μl of a solution of the minimum amount of WGA required to give agglutination, with dilutions of the inhibitors at the following concentrations: *A* and di-*N*-acetylchitobiose, 1 mg/ml; *B*, 15 mg/ml; GlcNAc, 10 mg/ml.

TABLE 2. *Challenge of BALB/c mice with two myeloma cell lines*

Tumor cell line	Schedule	Number of cells in challenge	Survival time of individual mice (days) Anti-*A* (MBSA)	Anti-*B* (MBSA)
XS63·5	I	10^7	24, 26	17, 19
		10^6	19, CR	11, 21
		5×10^5	CR, CR	22, 25
		10^5	CR, CR	CR, CR
MOPC 70A	II	10^6	29	24, 27
		10^5	34, 48	24, 31
		10^4	46, 57	31, 40

CR, complete regression.

WGA has a high degree of specificity for a structure present in *A*, but not in *B*. The hemagglutination-inhibition results further indicate that WGA is specific for a structure somewhat larger than the disaccharide, possibly as large as the structure presented in Fig. 1, and this structure is almost as large as the poly(L-asparate) antigenic determinant at the early stages of the immune response (19).

A was complexed with MBSA and the resulting complex, *A*(MBSA), was used as the antigen to evaluate immunization procedures in groups of mice. The corresponding *B*(MBSA) was used to immunize control groups of mice by identical procedures. The effectiveness of the immunization procedures was assessed by challenging control and immunized groups of mice with various numbers of cells of one of two BALB/c myeloma tissue-culture cell lines, XS63·5 and MOPC 70A (Table 2). When challenged with XS63·5 cells, the mice immunized against *A*(MBSA) by schedule I rejected at least five times as many tumor cells as control mice. Increased survival times in mice immunized against *A*(MBSA) by schedule II were observed for challenge with 10^4–10^6 MOPC 70A cells.

Tables 2 and 3 suggest that beyond some optimum conditions, immunization with more antigen and injections result in less protection. It may be that more vigorous immunization causes an enhancing humoral immune response that competes with cell-mediated protection. It should be noted that the minimum numbers of XS63·5 cells required to cause a tumor cannot be meaningfully compared from experiment to experiment, since this number is highly dependent on the age of the mice and state of the cells at the time of challenge.

The appearance of palpable tumors induced by 3-methylcholanthrene, which is known to induce localized tumors with a large degree of antigenic variation (3), is delayed in groups of mice immunized against *A*(MBSA), relative to unimmunized mice and mice immunized against *B*(MBSA) (Fig. 4). The close correspondence of the aged control and anti-*B*(MBSA) curves in Fig. 4*A* suggest that the amount of protection due to general immunological stimulation by complete Freund's adjuvant (20) is relatively small. A more vigorous immunization schedule was employed to produce the results presented in Fig. 4*B*; the decreased degree of protection observed suggests that under these conditions immunological enhancement of tumor growth may be competing with protection.

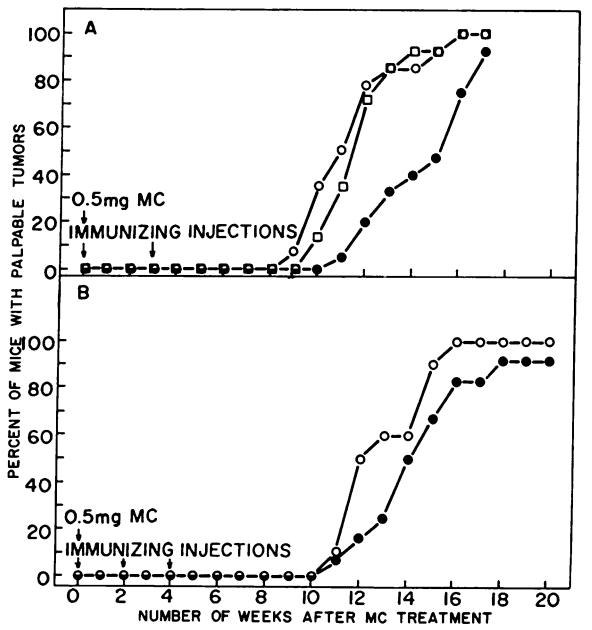

FIG. 4. Induction of tumors in immunized and control BALB/c mice by 3-methylcholanthrene (MC). On the day of initiation of the immunization schedules, each mouse received an intramuscular injection of 0.5 mg of MC in 0.1 ml of olive oil. (*A*) Appearance of palpable tumors in groups of mice immunized against 12.5 μg of *A*(MBSA) (●——●, 15 mice) or *B*(MBSA) (○——○, 14 mice) by immunization schedule IV, or unimmunized (□——□, 14 mice). (*B*) The appearance of palpable tumors in groups of mice immunized against 87.5 μg of *A*(MBSA) (●——●, 12 mice) by immunization schedule V, or unimmunized (○——○, 10 mice).

Two lines of evidence suggest that the observed protection is mediated by specific action of the immune system. First, some protection can be transferred from donor to unimmunized mice with spleen cells, while a degree of enhancement is transferred with serum (Table 4). Immunization schedule II, which does not stimulate a net protection in immunized mice (Table 3), was selected to produce a set of donors capable of donating both protection and enhancement of tumor growth to unimmunized recipients. Second, a single injection of 0.5 μg of *A*(MBSA) stimulates a degree of protection 3 weeks later (Table 3). This represents treatment with quantities of *A*(MBSA) substantially lower than would be expected to be required were it acting as a chemotherapeutic agent and than that required for general immunological stimulation by another polysaccharide, lentinan, which required 200 μg in 10 daily injections beginning at the time of challenge with tumor cells (21).

If the observation of Box *et al.* (7) that the WGA receptor site is present on untransformed fibroblasts in mitosis is true for all types of untransformed cells, immunization of an animal against this structure might cause destruction of normal dividing cells. Three phenomena that result from normal cell division were compared (Table 5) in groups of mice immunized against *A*(MBSA) and *B*(MBSA). Normal growth was evaluated by average weight gain during 8 weeks, and no adverse effects were noted. The humoral immune response in immunized mice was evaluated by the response to sheep erythrocytes measured by the plaque assay (12), the rosette assay (22), and the hemagglutination titer. The cellular immune response was evaluated by determining the survival time of C57Bl/6J skin allografts.

TABLE 3. *Effect of vigorous immunization against A(MBSA)*

Immunization procedure	Number of XS63·5 cells in challenge	Survival time of individual mice (days) Anti-*A* (MBSA)	Anti-*B* (MBSA)
0.5 μg, one injection (schedule VI)	10^7	9, 20	13, 20
	10^6	17, 21	18, 19
	5×10^5	CR, 26	25, 26
	10^5	CR, CR	CR, 39
10 μg, in two injections (schedule II)	10^7	19, 23	9, 16
	10^6	16, 24	19, 22
	5×10^5	30, 33	CR, 24
	10^5	CR, CR	CR, CR

CR, complete regression.

No marked effect on either immune system was observed, although the results suggest that a greater degree of suppression of cellular immune response occurs in mice immunized against *A*(MBSA). Wound healing was evaluated by comparing the rates of liver regeneration after partial hepatectomy. A small, but probably real, reduction in the rate of liver regeneration was observed.

DISCUSSION

The experiments employed a massive dose of a very potent carcinogen, which presumably transforms many normal cells in each mouse. Most are destroyed by the immune system, but even in immunized mice, transformed cells ultimately escape the immune system and develop into palpable tumors. The delay in appearance of palpable tumors in immunized mice perhaps reflects the greater number of transformants eliminated by the immune surveillance system when it is agumented by circulating memory cells (23) specific for the WGA receptor site.

TABLE 4. *Effect of spleen cells and serum transferred from immunized to unimmunized mice*

Number of XS63.5 cells in challenge	Survival time of individual recipients of spleen cells* or serum† from sensitized donors‡ (days) Anti-*A* (MBSA) spleen cells	Anti-*A* (MBSA) serum	Anti-*B* (MBSA) spleen cells	Anti-*B* (MBSA) serum
10^7	18, 18	16, 18	13, 15	10, 19
10^6	15, 17	14, 20	16, 17	17, 22
5×10^5	CR, CR	17, 19	18, 22	20, 23
10^5	CR, CR	19, 20	CR, CR	CR, 36
10^4	CR, CR	CR, CR	CR, CR	CR, CR

CR, complete regression.

* Unimmunized recipient mice received a single intraperitoneal injection of 3×10^8 viable spleen cells on the day before challenge.

† Unimmunized recipient mice received a single intraperitoneal injection of 0.25 ml of serum on the day before challenge.

‡ Donor mice were immunized by schedule II.

TABLE 5. *Effect of immunization against A(MBSA) on normal cell division*

Cell division phenomenon	Test system	Anti-*A* (MBSA)*	Anti-*B* (MBSA)*
Growth	Average weight gain during 8 weeks†	5.96 g	4.88 g
Immune response	No. of plaque-forming cells per 10^5 spleen cells‡	65	80
	No. of rosette-forming cells per 10^5 spleen cells‡	3520	6310
	Hemagglutination titer‡	1:64	1:64
	Average survival of C57B1/6J skin grafts§	11.2 ± 2 days	8.8 ± 1 days
Wound healing	Per cent of liver regenerated 3 days after partial hepatectomy¶	56%	67%

* Mice were immunized by schedule III. † Groups of 10 mice. ‡ Groups of 5 mice were immunized by an intraperitoneal injection of 0.2 ml of a 10% suspension of sheep erythrocytes in saline 5 days before assay. § Groups of about 10 mice received full-thickness pinch skin grafts under methofane anesthesia. ¶ The pooled results of about 50% hepatectomy (the two major lobes were ligated and excised under methofane anesthesia, and the incision was closed with sutures) evaluated at 3 days by total hepatectomy in weight-matched groups of 4 mice, and compared to the average weight of liver in groups of 5 weight-matched immunized control mice.

The observation of Box *et al.* (7) that the WGA receptor site is present on at least one type of cell in mitosis suggests that animals should be tolerant (23) to this antigenic determinant, since it is present during the development of the immune system and persists at low concentration throughout the life of the animal. Certainly no significant immune response is mounted against this site on tumor cells, since immunization against one tumor line affords little or no protection against others (3). Presentation of the antigenic determinant in a different form in *A*(MBSA) in the presence of complete Freund's adjuvant could break tolerance to the WGA site. Alternatively, the WGA sites on tumor cells may be nonimmunogenic for other reasons, and since they do not elicit a primary immune response, they might not elicit a secondary response in mice immunized against *A*(MBSA) when they are challenged with tumor cells. Experiments to determine how mice respond to the WGA site in each case are in progress.

In complement-deficient mice, such as the BALB/c mice used in these experiments, or in animals mounting only a cellular immune response to the WGA receptor site, attack on normal cells might be expected to occur only during rapid, localized cell division. Typically, a cell would be expected to pass through mitosis (about 1 hr) before any delayed hypersensitive response that it might stimulate could be carried out against it (about 18 hr). Only if there were another dividing cell nearby would the response be expected to continue and lead to cell destruction. These conditions may be met in liver regeneration.

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