Lysozyme Enhancement of Tumor Cell Immunoprotection in a Murine Fibrosarcoma

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ABSTRACT

We examined the ability of human lysozyme (HLZM) to enhance the immunogenicity of a methylcholanthrene-induced murine fibrosarcoma in C57BL/10 mice. Following s.c. injection of 10⁴ live tumor cells, 100% of mice developed palpable tumors within 16 ± 3 (S.D.) days. Prechallenge immunization with 10⁶ irradiated tumor cells with or without complete Freund's adjuvant resulted in protection from tumor development in 14 and 22% of mice, respectively. Incubation of tumor cells with HLZM prior to immunization approximately doubled the degree of protection, with 42 to 44% of mice remaining free of tumor. This enhanced protection was dependent on the enzymatic activity of HLZM. These data suggest that HLZM enhances tumor cell immunogenicity in this model.

INTRODUCTION

The development of syngeneic, inbred animal strains as model systems allowed the demonstration of tumor-associated transplantation antigens on the surfaces of tumor cells (7, 14). Tumor-associated transplantation antigens have been demonstrated on both carcinogen-induced and virally transformed animal tumors. Despite the presence of these antigens, most tumors continue to grow and eventually kill the host. Reasons for failure of immune defenses are unclear but have been attributed to the formation of blocking antibody (4), immune suppression, with HLZM prior to immunization approximately doubled the degree of protection, with 42 to 44% of mice remaining free of tumor. This enhanced protection was dependent on the enzymatic activity of HLZM. These data suggest that HLZM enhances tumor cell immunogenicity in this model.

MATERIALS AND METHODS

Animals. Female C57BL/10 mice, 8 to 12 weeks old, were obtained from The Jackson Laboratory, Bar Harbor, Maine. The animals were housed 10/cage and given food and water ad libitum.

Tumor. A sarcoma was induced by 3-methylcholanthrene and maintained in C57BL/10 mice by transplantation. To prepare single-cell suspensions, the solid tumors were excised and finely minced with a scalpel. The minced tumor tissue was then placed into 10 to 16 ml of RPMI Medium 1640:0.25% trypsin (1:1; both from Grand Island Biological Co., Grand Island, N.Y.) for 1 hr. The cell suspensions were periodically agitated to promote separation of the cell aggregates. The cells were washed 3 times and counted in a hemacytometer, and viabilities were determined by trypan blue exclusion. Cell suspensions were greater than 90% viable. Appropriate cell dilutions were made in the RPMI Medium 1640. All mice given s.c. inoculations of 10⁴ live tumor cells developed palpable tumors after 16 ± 3 (S.D.) days. The tumors grew to 90 to 110-mm diameter and killed the host at 35 ± 8 days after inoculation. No tumors regressed spontaneously, and all mice developing tumors eventually died of tumor progression.

Tumor Cell Irradiation. Single-cell suspensions of tumor were irradiated to 10,000 rads using a 60Co 4-MeV linear accelerator.

HLZM Treatment of Tumor Cells. HLZM was obtained from the urine of leukemic (monocytic or myelomonocytic) patients as described previously (13) and was the kind gift of Dr. Elliott Osserman, Columbia University, New York, N. Y. The preparations utilized in these studies formed a single band on sodium dodecyl sulfate: polyacrylamide gel electrophoresis and exhibited 4-fold more activity per mg in the standard Micrococcus lysodeikticus lytic assay than did egg white lysozyme. Irradiated tumor cells were suspended to 10⁹/ml in RPMI Medium 1640 and were incubated for 18 hr in the presence of HLZM (150 to 1000 µg/ml). Enzyme-treated tumor cells were not washed prior to injection into mice. Control or untreated cells were incubated for the same time interval. Tri-NAG was used to inhibit the enzymatic activity of HLZM in some experiments (11). Tri-NAG (300 µg/ml) completely inhibited the enzymatic activity of HLZM (160 µg/ml) for as long as 18 hr at 37°C. Tri-NAG was a generous gift of Dr. Gustav Lienhard, Dartmouth.
RESULTS

In an experiment to determine an appropriate challenge dose, injections of $10^5$ or more live tumor cells resulted in 100% tumor occurrence by an average of 19 ± 3 days (Table 1). This dose-response relationship was observed using both second- and fifth-passage cells. Incubation of mice with $10^5$ fifth-passage live tumor cells resulted in a 53% incidence of palpable tumor occurrence by 26 ± 4 days.

The immunogenicity of this methylcholanthrene-induced sarcoma was determined in 2 ways. Animals were immunized either by s.c. injection of $10^6$ irradiated cells or by injection of $10^6$ live tumor cells into the distal hind leg with amputation 7 days later. Fourteen days after immunization both groups were challenged with $10^2$, $10^4$, $10^5$, or $10^6$ live cells. In both experiments, protected animals were followed for at least 12 weeks to determine if a delay in tumor appearance rather than protection of the mice had occurred.

Table 1 shows the protection afforded by tumor immunization. All nonimmunized mice given injections of greater than $10^3$ live tumor cells developed tumors. Mice immunized 14 days prior to challenge with $10^6$ irradiated tumor cells were protected following live tumor cell challenge. The degree of protection decreased as the challenge dose increased. Tumor growth followed by excision produced similar immunoprotection. There may have been a loss of tumor cell immunogenicity or enhanced tumorigenicity between the second- and fifth-passage tumor cells, although a statistically significant difference ($p < 0.05$) is found only at the $10^6$ challenge dose. Tumor incidence in immunized mice ranged from 14 to 71% following challenge with $10^2$ to $10^6$ second-passage tumor cells and from 50 to 100% following fifth-passage tumor cell challenge. Immunization did not delay the appearance of tumors in either of these experiments. Protected animals did not develop tumors when followed for over 90 days.

To determine if HLZM could enhance immunogenicity, tumor cell suspensions were irradiated with 10,000 rads and then incubated for 18 hr at 37° with either medium alone, HLZM, or HLZM with its inhibitor, tri-NAG. Irradiated tumor cells were incubated at $1 \times 10^6$ cells/ml with HLZM at a final concentration of 150, 500, or 1000 µg/ml. Tri-NAG was added at a final concentration of 300 µg/ml. These different preparations, with and without complete Freund's adjuvant, were used to immunize mice. Fourteen days later, the mice were challenged with viable tumor cells. The animals were then followed for at least 12 weeks for occurrence of tumors.

Table 2 shows the enhanced protection afforded by HLZM treatment of immunizing tumor cells. Nonimmunized animals all developed tumors. Only 14 to 22% of mice immunized with non-HLZM-treated irradiated tumor cells were protected. A 2-fold increase in protection was demonstrated in the mice immunized with HLZM-treated tumor cells. The use of complete Freund's adjuvant did not alter the outcome. No difference in the degree of protection was seen with treatment of tumor cells using 3 different dosages of HLZM (150, 500, and 1000 µg/ml). Immunoprotection induced by HLZM-treated tumor cells was most effective when challenge dosages of $10^3$ and $10^4$ live tumor cells were used (Chart 1).

We then attempted to determine if the effect of HLZM on tumor cell immunogenicity was enzymatic (Table 3). Mice were immunized with $10^6$ non-HLZM-treated irradiated tumor cells or with $10^6$ HLZM-treated irradiated tumor cells. In one group of mice, $10^6$ irradiated tumor cells were incubated with HLZM and then washed before immunization. In 2 groups of mice, immunizing tumor cells were exposed to HLZM, but enzymatic activity on cells was prevented. In one of these groups, $10^6$ irradiated tumor cells were admixed with HLZM immediately prior to injection. In the second group, $10^6$ irradiated tumor cells were incubated with HLZM plus the inhibitor, tri-NAG. Control groups included nonimmunized mice or mice given injections of only HLZM or tri-NAG. All mice were challenged with $10^4$ live tumor cells 14 days after immunization. When the immunizing cells were pretreated with HLZM, 42% of the mice were protected. Removal of the HLZM by washing the cells after incubation did not decrease the degree of protection.
Nonenzymatic additions of HLZM to immunizing cells did not seem to enhance protection above that obtained with untreated cells.

**DISCUSSION**

Immunization with live or irradiated tumor cells protects mice to subsequent tumor cell challenge in this methylicholanthrene-induced sarcoma model. The degree of protection decreases with increased passage of tumor cells and with increased dosage of tumor cells. HLZM treatment of immunizing cells increased their protective capacity 2-fold after challenge with $10^5$ tumor cells. The additional use of complete Freund's adjuvant does not alter the immunoprotective efficacy of the immunizing preparations. The data suggest that the increase in protection afforded by HLZM-treated tumor cells is due to the enzymatic activity of HLZM.

That live irradiated tumor cells can immunize animals against subsequent challenge with viable syngeneic tumor cells has been well established, and our data are consistent with these observations (10). Furthermore, the changes in immunogenicity and tumorigenicity with cell passage and the dependency of immunoprotection on cell challenge dose have been observed previously (10).

HLZM pretreatment of immunizing tumor cells further enhanced their capacity to increase the resistance of mice to subsequent tumor challenge. This suggests that HLZM enhances tumor cell immunogenicity. These data are also consistent with our previous studies which demonstrated that lymphocytes pretreated with HLZM exhibit increased capacity to stimulate proliferation of allogeneic lymphocytes (15, 16). HLZM induces functionally important modifications of mammalian membrane receptors and glycoproteins (1, 6). The mechanism by which HLZM may modify tumor cells is unclear. However, a substrate of HLZM hydrolytic activity, the $\beta_1$-$4$ disaccharide of N-acetylglucosamine, is present in a variety of mammalian plasma and membrane glycoproteins (8, 9). The possibility that HLZM may alter tumor cell membranes is also supported by the observation that this enzyme modifies tumor cell morphology and proliferation kinetics in vitro (12). Our data supports the possibility that HLZM activity on tumor cells is enzymatic: (a) the effect was in part blocked by the presence of an enzymatic inhibitor of HLZM, tri-NAG; and (b) the effect was not observed if HLZM was not preincubated but instead injected as an admixture with tumor cells. Enzymatic alterations of a tumor cell surface may enhance immunogenicity by several possible mechanisms including alteration of antigens, "un-cloaking" cryptic antigens, or by changing surface charge or receptors so as to enhance cell-cell interaction. Insight into the mechanism of HLZM enhancement of immunogenicity would require detailed analysis of cell membrane glycoprotein structure of HLZM-treated cells and of alterations in immune response following immunization with HLZM-treated cells.

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